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13. ABSTRACT ***(Maximum 200 Words)***

Human Chk2 protein kinase is involved in DNA damage checkpoint pathway. After DNA damage Chk2 is phosphorylated at multiple sites in N-terminal SCD (SQ/TQ cluster domain) and activated. We reported here that Chk2 phosphorylated at T68 and T26/S28 is localized to the centrosome and the midbody. During mitosis we observed kinetochore localization of Chk2 phosphorylated at T26/S28. In attempt to find interactions between Chk2 and proteins with similar cellular localization, we found that Chk2 physically interacts with Plk1. Immunofluorescence has shown co-localization of phosphorylated Chk2 and Plk1 to the centrosome in early mitosis and to the midbody in late mitosis. We conclude that cellular localization of phosphoryalted Chk2 and its interaction with Plk1 are signs for a possible involvement in lateral communications between DNA damage and mitotic checkpoints.

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Introduction

Cell cycle checkpoints and DNA repair help to maintain genome stability. DNA damage cell cycle checkpoints arrest cells in their movement through the cell cycle to give them time for repair. Loss of the DNA damage checkpoints stimulates tumorigenesis and weakens the effect of chemotherapeutic agents. Inactivation of many of the genes involved in DNA damage checkpoints and DNA repair is linked to syndromes that cause a predisposition to cancer.

The protein kinase Chk2 is an important intermediary of vertebrate DNA damage checkpoint pathways. Chk2 is activated in response to DNA damage induced by ionizing radiation (IR), ultraviolet (UV) light, and by interference with DNA replication through a mechanism involving the phosphatidyl inositol (3') kinase-like kinases (PIKK) ATM (Ataxia-telangiectasia mutated) and ATR (1, 3). Upstream phosphorylation in response to DNA damage is required for activation of Chk2. Chk2 transmits the checkpoint signal to several downstream pathways leading to an arrest of the cell cycle in G1, S or G2/M phases. Mouse Chk2 -/- ES cells are defective in maintenance of G2 arrest and stabilization of p53 for initiation of G1 arrest. In response to DNA damage Chk2 phosphorylates p53, Cdc25A, Cdc25C, and Brca1 (reviewed in 4).

In addition to its importance in regulation of tumor suppressor genes *TP53* and *BRCA1*, *CHK2* is apparently a tumor suppressor gene itself. *CHK2* is defective in some families with variant Li-Fraumeni syndrome with wild type *TP53* (5). Heterozygous mutation to CHK2*1100delC increases the risk of breast cancer in women and men (6). Reports of *CHK2* mutations in sporadic and familial human cancers are accumulating (7, 8, 9, 10).

There is a small number of identified Chk2 targets and all are affected in human breast cancer. We hypothesized that other unidentified Chk2 substrates, as members of the same signaling network, will also be important for breast cancer biology. We will identify and characterize more Chk2 substrates. For this purpose we will generate several cell lines with altered Chk2 activities and their phenotype will be characterized. We will use them and alternative biochemical and genetic approaches to identified more Chk2 targets. Further we will characterize the interaction between Chk2 and its newly identified substrates.

Body

Our goal is to identify new Chk2 substrates. One of the approaches for this purpose is to establish cell lines with altered Chk2 activity. The technique of our choice was generation of a somatic cell line with a deletion of CHK2 gene. We have obtained the targeting vector from Dr. J. Sedivy and two genomic DNA clones containing CHK2 gene. In the process of subcloning of parts of CHK2 gene into the vector we had some difficulties that caused a delay. An additional concern was the publication showing that in the genome exist many CHK2 pseudogenes, which could be a reason for very low efficiency of the recombination of our targeting construct with the true CHK2 gene.

In aim 1B of the proposal we suggested, as an alternative approach for generation of cells with altered Chk2 activity, to generate cell lines stably expressing wild type or dominant-negative Chk2. For protein kinases, a mutant with defective kinase activity usually acts as dominant-negative protein. Expression of a dominant-negative mutant of Chk2 can inhibit endogenous wild type Chk2. To produce stable cell lines with

altered Chk2 activity we chose HT-1080 cells, which have TP53 gene mutated. They will be useful for studying p53-independent Chk2 down-stream events. We have transfected HT-1080 cells with pcDNA3-HA-Chk2 and pcDNA3-HA-Chk2D368A-KD containing a gene for neomycin resistance. Colonies of cells, stably transfected with the plasmid, were selected by treatment with an antibiotic-G418. Lysates from clones of G-418-resistant cells were tested by immunoblot (IB) with HA antibody for expression of HA-Chk2 and HA-Chk2D368A proteins.

To characterize those cell lines we tried to use phosphorylation of Ser20 after DNA damage as a measure of Chk2 activity. In my hands phosphospecific S20-p53 antibody from Cell Signaling Technology (CST) worked only with endogenous p53 from COS-7 cells but not with p53 from HT-1080, 293-T and U2OS. As an alternative we tested DNA damage-inducible p53 accumulation. We could not detect a difference in p53 accumulation after exposure to IR of HT-1080 cells stably expressing Chk2 and Chk2-KD. For pro of principle of the dominant-negative strategy, we performed co-transfection of U2-OS cells with HA-p53 and HA-Chk2 or HA-Chk2-KD. We observed that HA-p53 is accumulated after DNA damage only in the cells transfected with HA-Chk2 but not in the cells transfected with HA-Chk2-KD or empty vector.

To improve the assay we obtained from Dr. T. Hazalonetis a plasmid with p53IND, a mutant that can tetramerize with itself but not with endogenous wild type p53. In attempt to find a better cell line for our experiments we obtained HCT-15 cells from American Type Culture Collection. One of the*CHK2* alleles in these cells is mutated, total protein amount of Chk2 is low, and Chk2 is not activated after DNA damage. We plan to transfect stably HCT-15 cells with plasmids for wild type and kinase-dead *CHK2* genes.

We established a functional assay for the cell cycle effect of Chk2 activity. We performed FACS analysis of U2-OS cells, co-transfected with vectors for expression of GFP and HA-Chk2: WT or KD, 16 hours after exposure to 0 or 4 Gy IR. Results showed that twice more cells expressing Chk2-WT were arrested in G1 phase than cells expressing HA-Chk2-KD or empty vector.

Our second aim is screening for new Chk2 substrates. We made a list of potential Chk2 substrates on the base of genetic data received from yeast Chk2 homologs: Rad53 and Cds1. Another source for candidate substrates is a subset of proteins that undergo ATM-dependent phosphorylation after exposure of cells to IR. This list includes DNA polymerase α-primase, Dbf4-Cdc7, Plk1, Rpa, c-Abl, Mdm2, and Parp. We tested the relation of Chk2 with two of those proteins: replication protein A (RPA) and Polo-like kinase 1 (Plk1).

The middle subunit of replication protein A (Rpa2) undergoes phosphorylation after exposure to different DNA damaging agents. To test the possibility that Rpa2 is a Chk2 substrate we have performed a kinase assay with bacterially produced GST-Chk2 and purified Rpa, generously provided by Bruce Stillman's laboratory. We have found that GST-Chk2 phosphorylates the large (Rpa1) and middle (Rpa2) subunits of Rpa, but GST-Chk2-KD does not (Fig. 1A). We have also investigated, by co-immunoprecipitation, if Rpa and Chk2 can interact. We found that a small subpopulation of RPA2 co-immunoprecipitates with HA-Chk2 by IP with α-Chk2 and α-HA antibodies (Fig. 1B). Rpa is involved in DNA repair and a potential regulation by Chk2 could link DNA damage checkpoint pathway with DNA repair.

In attempt to identify new Chk2 interacting proteins we exploited the homology between yeast and mammalian DNA damage pathways. From genetic studies in yeast DNA damage checkpoint pathway it is known that RAD53 genetically interacts with CDC5, a gene for Polo-like kinase (11). After DNA damage Cdc5 is phosphorylated in Rad53-dependent manner (12). On the other hand CDC5 turns-off Rad53 in a process of adaptation to unrepairable DNA damage (13,14). The human homolog of CDC5 Polo-like kinase 1 is a multifunctional mitotic protein kinase involved in many mitotic events, including centrosome maturation and formation of the mitotic spindle (15). Also, Plk1 is a target of DNA damage checkpoint and it is inhibited after DNA damage in ATM-

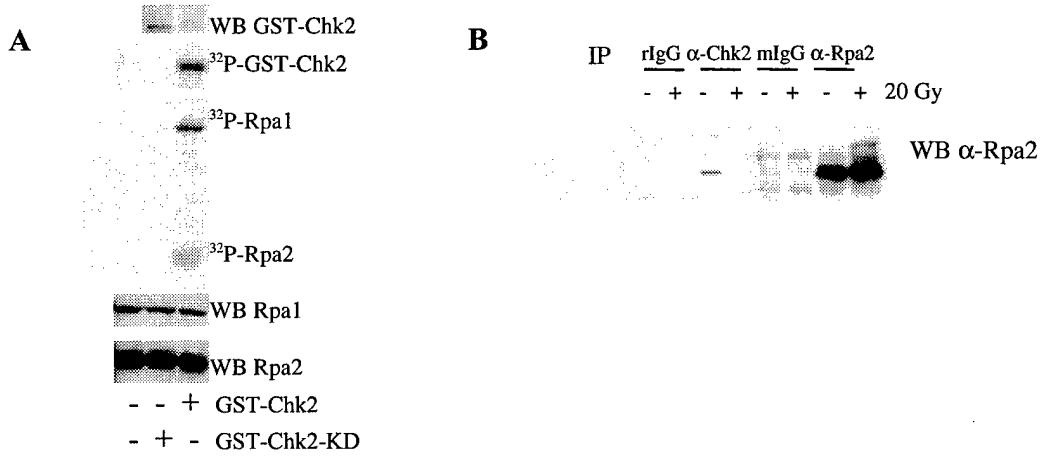

Figure 1. Chk2 interacts and phosphorylates RPA. (1A) GST-Chk2 phosphorylates Rpa1 and Rpa2 *in vitro*. (1B) Co-IP of Chk2 and Rpa2. Lysates from 293 cells transiently transfected with pcDNA3-HA-Chk2 were IP with α-Chk2, α-Rpa2, mIgG, and rIgG antibodies. Aliquots were run onto PAGE and IB with α-Rpa2.

dependent manner (16,17).

We examined by immunoprecipitation (IP) whether Chk2 and Plk1 interact. Extracts from 293-T cells, transiently transfected with plasmids for HA-tagged Chk2 and Flag-tagged Plk1, were IP with HA and Flag antibodies. Immunoblot (IB) analysis of the IPs showed that HA-Chk2 and Flag-Plk1 physically interact (Fig.4A, appendix 1).

To confirm this interaction we immunoprecipitatied Chk2 from whole cell extract from 293 cells and detected Plk1 in Chk2 immunoprecipitates by IB (Fig. 4B, appendix 1). Plk1 protein was more in IP from nocodazole-treated cells probably because of higher expression of Plk1 in mitotic cells (18). Chk2/Plk1 interaction was not affected by DNA damage introduced by treatment of cells with adriamycine, which cause Chk2 mobility shift and inhibition of Plk1 kinase activity toward α-casein (Fig.4B, appendix 1).

To characterize the interaction between Chk2 and Plk1 we created plasmids expressing deletion mutants of HA-Chk2 and Flag-Plk1. Mutant HA-Chk2 and Flag-Plk1 proteins were expressed in 293-T cells and co-IP with HA and Flag antibodies were performed. The data from IB analysis of immunoprecipitates showed modulation of Chk2/Plk1 interaction, although we could not determine a single domain in the proteins critically important for their physical interaction. This fact could be explained by a more complicated anatomy of the Chk2/Plk1 complex.

To investigate further the interaction between Chk2 and Plk1, we tested if they co-localized in the cell. Plk1 is expressed in S, G2 and M phases of cell cycle and its activity peaks in M phase. Plk1 is associated with the kinetochores of condensed chromosomes, the centrosome in prophase and metaphase, and the midbody, later in mitosis. From the work of others it was known that Chk2 is localized mainly in the nucleus. Phosphorylation of Chk2 at several SQ/TQ sites in its N-terminal part is required for Chk2 activation after DNA damage. ATM prefers one of this sites-Thr68 which phosphorylation is required for Chk2 DNA damage-dependent activation (19). To track the location of active Chk2 we raised and affinity purified a phospho-specific antibody, raised against phosphopeptide resembling the region around phosphoThr68, that recognized Chk2 only when it is phosphorylated at Thr68. The specificity of the antibody was examined by WB analysis. PT68-Chk2 recognized endogenous Chk2 and transiently expressed HA-Chk2 from 293-T cells irradiated with IR but not from mock-irradiated cells. The antibody did not recognize transiently expressed mutant HA-Chk2T68A neither from irradiated nor from non-irradiated cells (Fig. 1A, appendix 1).

At the sites of DNA damage, Chk2 is phosphorylated at Thr68 and formed immunoreactive foci. To test if our antibody can detect these foci we immunostained 293-T, WI-38, HT-1080 and GM5849C cells irradiated with 0 Gy or 4 Gy gamma radiation. We observed foci formation following exposure to IR in 293-T, WI-38 and HT-1080 cells but not in GM5849C cells, an AT cell line (Fig. 2D, appendix 1).

Immunofluorescence (IF) of 293 cells with PT68-Chk2 antibody produced a staining pattern characteristic of proteins associated with centrosomes (Fig. 2A, appendix 1). Signals generally consisted of single or paired nuclear dots in interphase cells, separated dots flanking condensed chromatin in metaphase, and single dots adjacent to chromatin in telophase (Fig. 2A, appendix 1). Similar staining patterns with α-PT68-Chk2 were observed with three additional cell lines: WI38, HT-1080, U2OS and GM5849C (AT cells). The signal is ATM-independent since it was similar in ATM-deficient GM5849C cells.

Additional controls were performed to verify specificity of the antibody. IF background was low with control nonspecific IgG as primary antibody. Competition with the oligophosphopeptide used as antigen for production of PT68-Chk2 antibody eliminated the IF signal (Fig. 2C, appendix 1). γ-tubulin nucleates microtubule assembly, and is concentrated at centrosomes (20). To confirm the localization of Chk2 to the centrosome, we performed double IF using α-PT68-Chk2 and α-γ-tubulin antibodies in 293 cells (Fig. 2B, appendix 1). The PT68-Chk2 and γ-tubulin fluorescence signals overlapped at the strong, centrosome-like foci seen with α-PT68-Chk2, providing direct evidence for centrosomal localization of phosphorylated Chk2 (Fig. 2 B, C). However, in contrast to PT68-Chk2 signals, the γ-tubulin signal was not competed with the PT68-containing phosphopeptide (Fig. 2C, appendix 1). In order to verify that the α-PT68-Chk2 signal is a result of Chk2 phosphorylation, rather than high local concentration of Chk2 or other artifact, we treated permeabilized U2-OS cells with λ-phosphatase. Phosphatase treatment eliminated the PT68-Chk2 signal, but the γ-tubulin signal remained although slightly reduced (Fig. 2D, appendix 1). Incubation with buffer did not change either γ-tubulin nor PT68-Chk2 signals.

In addition to centrosome-associated staining, we detected phosphorylated Chk2 with α-PT68-Chk2 and α-PT26/PS28-Chk2 antibodies at two other sites. First, in telophase, they were concentrated at the midbody, the central part of the cytokinetic bridge (Fig.3B, appendix 1). Immunoreactive phospho-Chk2 was also found in discrete dots of condensed chromosomes in U2-OS cells imunnostained with α-PT26/PS28-Chk2 antibody (Fig. 3A, appendix 1). This staining pattern indicates that Chk2 might associate with chromosomal kinetochores or centromeres. This signal co-localized with the positions of CENP-E protein, a known kinetochore marker (Fig. 3C, appendix 1). Chk2 phosphorylated at S26/28 Chk2 was detected at kinetochores in mitosis (Fig. 3A, C, appendix 1).

To study co-localization of Chk2 and Plk1 we co-immmunostained 293-T and U2OS cells with PT68-Chk2 and Plk1 antibodies. Early in mitosis (prophase and metaphase) Plk1 and PT68-Chk2 were associated with the centrosomes. Later in mitosis PT68-Chk2 signal split between the centrosomes and the midbody while Plk1 signal was localized only to the midbody (Fig. 5, appendix 1). As it was described above PT26/PS28-Chk2 associated with kinetochores and GFP-Plk1 localizes to kinetochores (21).

We have found that subsets of Chk2, phosphorylated at sites required for DNA damage responses, and co-localize with centrosomes, midbodies, and kinetochores. The results are surprising, because numerous studies have implicated Chk2 and its yeast homologs in DNA damage responses and DNA replication checkpoints, but Chk2 has not been directly linked to activity of checkpoints that detect assembly of the mitotic spindle or its attachment to kinetochores. Potential activities of Chk2 at these sites include normal or checkpoint regulation of the centrosome cycle, spindle attachment, and/or cytokinesis. Alternatively, phospho-Chk2 could provide lateral communication between these processes and DNA damage and replication inhibition responses.

Key Research Accomplishments:

- New Chk2 substrates were found-Rpa1 and Rpa2.
- A physical interaction between Chk2 and Polo-loke kinase was detected.
- New cellular locations of phosphorylated Chk2 were observed: centrosomes, midbody, and kinetochores.
- Co-localization of Plk1 and phosphorylated Chk2 to the centrosomes and the midbody.

Reportable Outcomes:

1.) Cellular localization of phosphorylated Chk2 - **Lyuben Tsvetkov**, Xingzhi Xu, Jia Li, and David F. Stern Submitted manuscript.

2.) "Chk2 Activation and Phosphorylation-Dependent Oligomerazation" X. Xu, **L. Tsvetkov**, and D. F. Stern 2002. Mol . Cell Biol. 22:4419-4432.

Conclusions

Establishment of Chk2-/- cells was delayed because of technical difficulties. Cell lines with stable expression of Chk2 wild type and dominant negative mutant were produced. Functional assays for Chk2 effects on cell cycle by FACS analysis and p53 protein levels were developed. These assays will be improved through using of more suitable cell lines and p53 mutants. We have found new substrates of Chk2 by *in vitro* kinase assay: the large and middle subunits of RPA. A new Chk2 interacting protein, Polo-like kinase 1 was detected by co-IP of endogenous and exogenous epitope-tagged Chk2 and Plk1. The interaction is not DNA damage regulated. Binding assays with deletion Chk2 and Plk1 mutants have not shown a single domain in Chk2 and Plk1 critical for this interaction. IF of cells from several cell lines with anti-phospho-T68-Chk2 antibody revealed a localization of phosphorylated Chk2 to the centrosomes and the midbody. Also, we have observed localization of phosphorylated Chk2 to the kinetochores using another phosphospecific antibody - anti-PT26/PS28-Chk2. Dual immunostaining with anti-Plk1 and anti-phospho-Chk2 antibodies indicated for co-localization to the centrosome and the midbody. For fulfillment of the aims of the proposal further characterization of Chk2/Plk1 interaction and regulation will be done. The significance of RPA phosphorylation by Chk2 needs to be determined. Cell lines with altered Chk2 activity will be improved and characterized further.

References

1. Matsuoka, S., Huang, M., and Elledge, S. J. (1998) *Science* **282**(5395), 1893-7.
2. Brown, A. L., Lee, C. H., Schwarz, J. K., Mitiku, N., Piwnica-Worms, H., and Chung, J. H. (1999) *Proc Natl Acad Sci U S A* **96**(7), 3745-50.
3. Chaturvedi, P., Eng, W. K., Zhu, Y., Mattern, M. R., Mishra, R., Hurle, M. R., Zhang, X., Annan, R. S., Lu, Q., Faucette, L. F., Scott, G. F., Li, X., Carr, S. A., Johnson, R. K., Winkler, J. D., and Zhou, B. B. (1999) *Oncogene* **18**(28), 4047-54.
4. Bartek, J., Falck, J., and Lukas, J. (2001) *Nat Rev Mol Cell Biol* **2**(12), 877-86.
5. Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., Lubratovich, M., Verselis, S. J., Isselbacher, K. J., Fraumeni, J. F., Birch, J. M., Li, F. P., Garber, J. E., and Haber, D. A. (1999) *Science* **286**(5449), 2528-31.
6. Meijers-Heijboer, H., Van Den Ouweland, A., Klijn, J., Wasielewski, M., De Snoo, A., Oldenburg, R., Hollestelle, A., Houben, M., Crepin, E., Van Veghel-Plandsoen, M., Elstrodt, F., Van Duijn, C., Bartels, C., Meijers, C., Schutte, M., McGuffog, L., Thompson, D., Easton, D. F., Sodha, N., Seal, S., Barfoot, R., Mangion, J., Chang-Claude, J., Eccles, D., Eeles, R., Evans, D. G., Houlston, R., Murday, V., Narod, S., Peretz, T., Peto, J., Phelan, C., Zhang, H. X., Szabo, C., Devilee, P., Goldgar, D., Futreal, P. A., Nathanson, K. L., Weber, B. L., Rahman, N., and Stratton, M. R. (2002) *Nat Genet* **31**(1), 55-9.
7. Matsuoka, S., Nakagawa, T., Masuda, A., Haruki, N., Elledge, S. J., and Takahashi, T. (2001) *Cancer Res* **61**(14), 5362-5.
8. Hofmann, W. K., Miller, C. W., Tsukasaki, K., Tavor, S., Ikezoe, T., Hoelzer, D., Takeuchi, S., and Koeffler, H. P. (2001) *Leuk Res* **25**(4), 333-8.
9. Ingvarsson, S., Sigbjornsdottir, B. I., Huiping, C., Hafsteinsdottir, S. H., Ragnarsson, G., Barkardottir, R. B., Arason, A., Egilsson, V., and Bergthorsson, J. T. (2002) *Breast Cancer Res* **4**(3)

10. Miller, C. W., Ikezoe, T., Krug, U., Hofmann, W. K., Tavor, S., Vegesna, V., Tsukasaki, K., Takeuchi, S., and Koeffler, H. P. (2002) *Genes Chromosomes Cancer* **33**(1), 17-21.
11. Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M., and Elledge, S. J. (1999) *Science* **286**(5442), 1166-71.
12. Cheng, L., Hunke, L., and Hardy, C. F. (1998) *Mol Cell Biol* **18**(12), 7360-70.
13. Toczyski, D. P., Galgoczy, D. J., and Hartwell, L. H. (1997) *Cell* **90**(6), 1097-106.
14. Pellicioli, A., Lee, S. E., Lucca, C., Foiani, M., and Haber, J. E. (2001) *Mol Cell* **7**(2), 293-300.
15. Lane, H. A., and Nigg, E. A. (1996) *J Cell Biol* **135**(6 Pt 2), 1701-13.
16. Smits, V. A., Klompmaker, R., Arnaud, L., Rijksen, G., Nigg, E. A., and Medema, R. H. (2000) *Nat Cell Biol* **2**(9), 672-6.
17. van Vugt, M. A., Smits, V. A., Klompmaker, R., and Medema, R. H. (2001) *J Biol Chem* **276**(45), 41656-60.
18. Nigg, E. A. (1998) *Curr Opin Cell Biol* **10**(6), 776-83.
19. Zhou, B. B., Chaturvedi, P., Spring, K., Scott, S. P., Johanson, R. A., Mishra, R., Mattern, M. R., Winkler, J. D., and Khanna, K. K. (2000) *J Biol Chem* **275**(14), 10342-8.
20. Schiebel, E. (2000) *Curr Opin Cell Biol* **12**(1), 113-8.
21. Arnaud, L., Pines, J., and Nigg, E. A. (1998) *Chromosoma* **107**(6-7), 424-9.

Appendices

1.) Cellular localization of phosphorylated Chk2 - **Lyuben Tsvetkov**, Xingzhi Xu, Jia Li, and David F. Stern Submitted manuscript.

2.) "Chk2 Activation and Phosphorylation-Dependent Oligomerazation" X. Xu, **L. Tsvetkov**, and D. F. Stern 2002. Mol . Cell Biol. 22:4419-4432.

Cellular localization of phosphorylated Chk2

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Abstract

The protein kinase Chk2 is an intermediary in DNA checkpoint pathways. Activation of Chk2 by DNA damage involves phosphorylation at specific sites. Phospho-specific antibodies were used to localize active forms of Chk2 by immunofluorescence. Chk2 phosphorylated at T68 and T26 or S28 is co-localized with the centrosome and the midbody. During mitosis, Chk2 phosphorylated at T26 and/or S28 co-localized with kinetochores. Another protein with similar subcellular localization patterns, Plk1, was found to physically interact with Chk2. These results may signify involvement of Chk2 in communication between DNA damage and mitotic checkpoints.

Introduction

The protein kinase Chk2 is an important intermediary in vertebrate DNA damage checkpoint pathways (reviewed in Bartek et al., 2001). Chk2 is activated by several forms of DNA damage and by interference with DNA replication. Activation of Chk2 is associated with Chk2 phosphorylation. Chk2 is activated through a mechanism primarily involving the phosphatidyl inositol (3') kinase-like kinase (PIKK) ATM (Ataxia-telangiectasia mutated) in response to double-strand DNA breaks (DSBs) induced by ionizing radiation (IR)(Matsuoka et al., 1998). Activation of Chk2 by other forms of DNA damage or replicational stress can occur independent of ATM, probably through activity of the related PIKK ATR (Brown et al., 1999; Chaturvedi et al., 1999; Matsuoka et al., 1998). Chk2 transduces checkpoint signals to downstream pathways leading to arrest of the cell cycle in G1, S or G2/M phases through phosphorylation of p53, Cdc25A, and Cdc25C (Chehab et al., 2000; Shieh et al., 2000; Falck et al., 2001; Matsuoka et al., 1998). Chk2-dependent phosphorylation of Brca1 plays a protective role after DNA damage (Lee et al., 2000). In addition to its involvement in regulation of tumor suppressor genes TP53 and BRCA1, CHK2 is itself a tumor suppressor gene (Bell et al., 1999; Hofmann et al., 2001; Ingvarsson et al., 2002; Matsuoka et al., 2001; Miller et al., 2002).

Results and discussion

Damage-dependent activation of Chk2 is accompanied by phosphorylation of sites in a cluster, called the SCD (SQ/TQ cluster domain), which consists of a series of SQ/TQ sites near the amino terminus of Chk2 (Matsuoka et al., 2000). These sites are consensus targets for phosphorylation by PIKKs, including the Chk2 regulators Atm and Atr, and their phosphorylation is associated with Chk2 activation.

Although Chk2 is activated by DNA damage, little is known about regulation of Chk2 during the normal cell cycle. Phosphorylation of Chk2 at one or more of the SCD sites is required for Chk2 activation after DNA damage (Ahn et al., 2000; Matsuoka et al., 2000; Melchionna et al., 2000; Zhou et al., 2000). Since DNA damage-dependent activation of Chk2 requires Chk2 phosphorylation, we have used phospho-specific antibodies directed against sites within the SCD to probe for possible sites of Chk2 activity in non-damaged cells. One antibody recognizes phospho-T68 (αPT68-Chk2), which is the predominant site phosphorylated in response to DSBs, and is required for intact responses to DNA damage (Ahn et al., 2000; Zhou et al., 2000). ATM is the major Chk2 regulator in this response, and preferentially phosphorylates T68 *in vitro* (Matsuoka et al., 2000). In response to replication blockade or UV light, this site is phosphorylated independent of ATM, probably by ATR (Matsuoka et al., 2000). A second antibody was raised against a peptide containing phospho-T26 plus phospho-S28 (αPT26/PS28-Chk2). Although S28 is not a good substrate for either ATM or ATR *in vitro*, T26 is a good ATR substrate *in vitro* (Matsuoka et al., 2000).

Characterization of αPT68-Chk2 antibody.

In irradiated 293-T cells, but not mock-irradiated cells, αPT68-Chk2 recognized endogenous Chk2 (lower band, Fig. 1A, lanes 2 and 4) and transiently-expressed HA-Chk2 (upper band, Fig. 1A, lane 2). However, this antibody did not recognize transiently expressed HA-Chk2T68A, in which the target phosphorylation site has been replaced with alanine (Fig. 1A, lane 4). In 293-T cells exposed to increasing doses of IR, αPT68-Chk2 antibody recognized endogenous Chk2 as a single band that increased in intensity with escalating radiation dose, while anti-Chk2 antibody (αChk2) detected equal amounts of Chk2 protein in all samples (Fig.1B). Recombinant Chk2 undergoes autophosphorylation at multiple sites when expressed in bacteria (Xu et al., 2002). αPT68-Chk2 recognized GST-Chk2-WT, but not GST-Chk2D368A, which has a mutation that eliminates kinase catalytic activity (Fig. 1C).

Focal nuclear localization of phospho-Chk2 after DNA damage was documented in immunofluorescence (IF) experiments using a different preparation of phospho-specific antibodies directed against Chk2 PT68 (Ward et al., 2001). Similarly, we found that PT68-Chk2 immunoreactive foci are formed following exposure to IR in 293-T and HT-1080 cells, but not in GM5849C cells, which lack functional ATM (Fig. 1D).

Phosphorylated Chk2 is localized to the centrosome.

IF of 293 cells with αPT68-Chk2 antibody revealed a staining pattern characteristic of proteins associated with centrosomes (Fig. 2A). Signals generally consisted of single or paired nuclear dots in interphase cells, separated dots flanking condensed chromatin in metaphase, and single dots adjacent to chromatin in telophase (Fig. 2A). The signal co-localizes with anti-γ-tubulin (αγ-tubulin) IF (Fig. 2 B, C), providing evidence for centrosomal localization of phosphorylated Chk2 (Schiebel,

2000). Similar staining patterns with αPT68-Chk2 were observed with three additional cell lines: WI38, HT-1080, U2-OS and GM5849C (AT cells) (LT, unpublished data). The signal is at least partly ATM-independent since it was similar in ATM-deficient GM5849C cells.

Additional controls were performed to verify specificity of αPT68-Chk2. IF background was low when control nonspecific IgG was used as primary antibody (unpublished data). Competition with the antigenic oligophosphopeptide used as antigen for production of αPT68-Chk2 eliminated the αPT68-Chk2, but not the αγ-tubulin IF signal (Fig. 2C). λ-phosphatase treatment of permeabilized U2-OS cells eliminated the αPT68-Chk2 signal, but only slightly reduced the γ-tubulin signal, consistent with recognition of a phosphoepitope by the former (Fig. 2D). Incubation with phosphatase buffer did not affect either γ-tubulin nor PT68-Chk2 signals (unpublished data).

The majority of Chk2 in cultured cells is not phosphorylated and is not recognized by αPT68-Chk2 (Fig. 1A, B). The simplest interpretation of the ability to detect punctate IF with αPT68-Chk2 is that a minor subpopulation of Chk2 is phosphorylated. IF with αChk2 detected a diffuse nuclear and cytoplasmic localization that overlapped with focal αPT68-Chk2 fluorescence at centrosomes (Fig. 2E).

IF with αPT26/PS28-Chk2 antibody.

Treatment of 293 and U2-OS cells with IR, UV, or hydroxyurea induced immunoreactivity of exogenously-expressed HA-Chk2 with αPT26/PS28-Chk2 antibody analyzed by immunoblotting (IB). The antibody detects endogenous Chk2 in cells exposed to IR or adriamycin (unpublished data). Also, it recognizes wild-type HA-Chk2 but not HA-Chk2T26A/S28A with alanine substitutions at the immunogenic sites (XX, unpublished data). Similar to αPT68-Chk2, IF with αPT26/PS28-Chk2 in U2-OS cells yielded a centrosome-like pattern (Fig. 3 A, B).

Chk2 is co-localized with kinetochores and the midbody.

In addition to centrosome-associated staining, we detected phosphorylated Chk2 with αPT68-Chk2 and αPT26/PS28-Chk2 antibodies at two other sites. In telophase, they were concentrated at the midbody (Fig. 3B). Immunoreactive phospho-Chk2 was also found in discrete dots of condensed chromosomes in U2-OS cells immunostained with αPT26/PS28-Chk2 antibody (Fig. 3A), suggestive of centromere or kinetochore localization. The signal co-localized with the staining of αCENP-E (Fig. 3C), a known kinetochore marker. Chk2 phosphorylated at T26/S28 was detected at kinetochores in mitosis (Fig. 3A, C). Before anaphase onset (prophase and metaphase) the signal was stronger than in anaphase and telophase (LT, unpublished data). We did not observe phospho-T26/S28 Chk2 at kinetochores during interphase although it was present at centrosomes.

Chk2 interacts with Plk1.

Multiple cell cycle regulatory proteins localize to centrosomes and kinetochores, including CDK regulators and checkpoint proteins (reviewed in Skibbens and Hieter, 1998; Lange, 2002). Members of the polo-like kinase (PLK) family are involved in cell cycle regulation during mitosis, including positive regulation of mitotic entry, anaphase progression and mitotic exit. Plk1 is expressed in S, G2 and M phases of the cell cycle and its activity peaks in M phase. Like phospho-Chk2, PLKs are localized to centrosomes, kinetochores, and midbodies (reviewed in Nigg, 1998). Antagonistic

genetic interactions between the yeast Chk2 homolog Rad53 and the PLK Cdc5 have been identified (Sanchez et al., 1999; Toczyski et al., 1997; Pellicioli et al., 2001; Hu et al., 2001). Since these results suggested communication between PLK and Chk2 pathways, we determined whether Chk2 and Plk1 physically interact. HA-Chk2 and Flag-Plk1 co-immunoprecipitate when jointly expressed by transient transfection (Fig.4A, lanes 3, 6), and endogenous Chk2 and Plk1 also co-precipitate (Fig. 4B, lanes 3, 4). The recovery of Plk1 protein in immunoprecipitates from nocodazole-treated cells was greater (Fig. 4B, lanes 9, 10), which may simply reflect the higher expression of Plk1 in mitotic cells (reviewed in Nigg, 1998). Similarly, the Chk2/Plk1 co-IP was not affected by DNA damage caused by treatment of cells with adriamycin (Fig. 4B, lane 4). However, adriamycin induced Chk2 mobility shift associated with phosphorylation (Fig. 4B, lanes 2, 4), and inhibited *in vitro* Plk1 kinase activity toward α-casein (Fig. 4B, line 10).

Chk2 and Plk1 co-localize to the centrosome and the midbody.

Plk1 is associated with the kinetochores of condensed chromosomes, the centrosome in prophase and metaphase, and the midbody, later in mitosis (Arnaud et al., 1998; Golsteyn et al., 1995). To investigate possible co-localization of Chk2 and Plk1 we performed dual IF of 293-T and U2-OS cells with αPT68-Chk2 and αPlk1 antibodies. Early in mitosis (prophase and metaphase) Plk1 and PT68-Chk2 were apparently associated with the centrosomes (Fig. 5A, B). Later in mitosis, PT68-Chk2 was detected at the centrosomes and the midbody, whereas the Plk1 signal was localized only to the midbody (Fig. 5C). This suggests that Plk1 is not required to maintain centrosomal localization of Chk2. As described above, PT26/PS28-Chk2 associates with kinetochores, and GFP-Plk1 localizes to kinetochores (Arnaud et al., 1998).

We have found that subsets of Chk2, phosphorylated at sites required for DNA damage responses, and co-localize with centrosomes, midbodies, and kinetochores. The results are surprising, because numerous studies have implicated Chk2 and its yeast homologs in DNA damage responses and DNA replication checkpoints, but Chk2 has not been directly linked to activity of checkpoints that detect assembly of the mitotic spindle or its attachment to kinetochores. Potential activities of Chk2 at these sites include normal or checkpoint regulation of the centrosome cycle, spindle attachment, and/or cytokinesis. Alternatively, phospho-Chk2 could provide lateral communication between these processes and DNA damage and replication inhibition responses.

Although the Chk2 SQ/TQ sites monitored in these experiments are sites for PIKK-dependent phosphorylation in the DNA damage response, these same sites may also be targets for PIKK-independent phosphorylation. Chk2 expressed in E.coli can trans-phosphorylate kinase-defective Chk2 at T68 (Xu et al., 2002) and T26 or S28 (XX, unpublished data). The finding that at least one conventional (non-PIKK) Ser/Thr kinase, Chk2 itself, can phosphorylate this site suggests that, in centrosomes and other sites associated with phospho-Chk2, other protein kinases may be responsible for Chk2 phosphorylation. Recruitment of Chk2 to centrosomes and kinetochores could be mediated through binding of the FHA domain of Chk2 to phosphoproteins located there. This may deliver Chk2 in close proximity to activating kinases at these sites. High local concentrations of Chk2 may also enable Chk2-Chk2 transphosphorylation, which may, in turn, be sufficient to activate Chk2 (Xu et al., 2002; Ahn et al., 2002). Non-PIKK Ser/Thr kinases localized to centrosomes include Aurora family kinases (reviewed in Descamps and Prigent, 2001), and at some phases of the cell cycle, checkpoint kinases Mps1, Bub1,

and BubR1 (reviewed in Gillett and Sorger, 2001). Cell-cycle-dependent localization of Plk1 to the centrosomes, kinetochores and midbody has been reported (Arnaud et al., 1998; Golsteyn et al., 1995).

Proper regulation of centrosome duplication and maturation is vital for maintenance of a bipolar mitotic spindle. Aberrant centrosome and spindle numbers are common hallmarks of aggressive cancers. Mutations in genes associated with DNA damage responses, including p53, have been implicated in aberrant partitioning of centrosomes after cell division, and genomic instability (Fukasawa et al., 1996). Centrosome amplification in tumor and cell lines is linked to mutations in p53 (Fukasawa et al., 1996), abnormal expression of MDM2 (Carroll et al., 1999) and p21CIP1 (Mantel et al., 1999). The mechanisms for these changes are not completely understood, and may reflect direct regulation of centrosome maturation and stability through these pathways, or indirect effects on survival of changes resulting in multinucleation and polyploidy (Meraldi et al., 2002). The Chk2 substrate BRCA1, partly co-localizes with Chk2 under basal conditions (Lee et al., 2000), and displays centrosomal localization (Hsu and White, 1998). Brca1 disassociation from centrosomes could be phosphorylation dependent and it is interesting the localization of Brca1 mutated at the site of Chk2 phosphorylation. Mutations in the DNA repair protein Brca2 (Tutt et al., 1999) or overexpression of the Chk2 kinase ATR (Smith et al., 1998) also affect centrosome number.

Immunoreactive phospho-T26/S28 Chk2 co-localized with kinetochores. Kinetochores have an active role in the spindle checkpoint that delays anaphase onset until all chromosomes are attached to the spindle. Several proteins involved in this checkpoint are also localized to kinetochores and/or centrosomes: Mad1, Mad2, Mad3, Bub1, Bub3, hBubr1, and Mps1 (reviewed in Skibbens and Hieter, 1998; Jullien et al., 2002). In view of the physical association between Chk2 and Plk1, it is interesting that GFP-Plk1 also localizes to kinetochores in mitosis (Arnaud et al., 1998). Another DNA damage checkpoint protein, 53BP1, has recently been reported to localize to the kinetochore during mitosis (before anaphase) (Jullien et al., 2002).

Results in yeast systems suggested that Chk2 interacts antagonistically with PLKs (Sanchez et al., 1999). Plk1 is a target of the DNA damage checkpoint and is inhibited after DNA damage in an ATM-dependent manner (Smits et al., 2000; van Vugt et al., 2001). After DNA damage, Cdc5 is phosphorylated in Rad53-dependent manner (Cheng et al., 1998). CDC5 inhibits Rad53 in the process of adaptation to unrepairable DNA damage (Toczyski et al., 1997; Pellicioli et al., 2001), and was implicated as a possible Rad53 effector (Sanchez et al., 1999). Recent work suggests that Cdc5 and Rad53 antagonistically regulate mitotic pathways through the common regulatory target Bfa1 (Hu et al., 2001). It is noteworthy that Bfa1 is located at spindle pole bodies, where it regulates activity of *TEM1*, the limiting regulator of CDK inactivation required for mitotic egress. Perhaps localization of Chk2 to centrosomes reflects interaction with a similar mitotic exit complex.

The unexpected localization of phosphorylated forms of Chk2 to centrosomes, kinetochores, and midbodies, suggest hitherto unexplored connections of these DNA checkpoint pathways with chromosome mechanics. Since centrosome and spindle aberrations are common in tumor cells with dysfunctional checkpoint pathways, these findings may shed new light on genomic instability.

Materials and methods

Cell cultures, transfections and treatments

ATM-deficient (GM5849C) human fibroblasts were obtained from Coriell Institute for Medical Research, Camden, N.J. Other cell lines were obtained from American Type Culture Collection. Cells were transfected by calcium phosphate precipitation with 5 μg of plasmid DNA and were lysed after 40 h. Cells were treated with nocodazole (Sigma) (250ng/ml) for 16 h and adriamycin (Sigma)(0.5μM) for 1h. Cells were irradiated in a Mark I ^{137}Cs irradiator (Shepard).

Antibodies

Rabbit polyclonal αPT68-Chk2 (Xu et al., 2002) was affinity purified by phosphopeptide affinity chromatography. Non-phosphospecific rabbit polyclonal αChk2 antibody was produced by immunization with recombinant GST-Chk2 produced in E.coli. Mouse αPlk1 mAb cocktail was obtained from Zymed Laboratories; mouse αChk2 mAb from Neomarkers; and affinity purified rabbit polyclonal αPT26/PS28 antibody was a generous gift of Yi Tan (Cell Signaling Technology). Mouse αγ-tubulin mAb (clone GTU-88), mouse αFlag mAb were obtained from Sigma; polyclonal goat αCENP-E (C-19) from Santa Cruz Biotechnology; mouse anti-hemagglutinin (HA) mAb (16B12) from BabCo/Covance; horseradish peroxidase (HRP)-conjugated rat anti-HA mAb (3F10) from Roche Molecular Biochemicals; and HRP-conjugated secondary antibodies were obtained from Pierce.

Plasmids

pcDNA-HAChk2 and pcDNA-HAChk2T68A are described in (Xu et al., 2002). Plk1 coding sequence was from an EST clone (GenBank accession no. BE 900300, obtained from Research Genetics) was amplified by polymerase chain reaction and cloned into pcDNA3-3xFlag vector, resulting in pcDNA-FlagPlk1. pGEX2TK-GSTChk2 and pGEX2TK-GSTChk2 (D368A) described in (Xu et al., 2002) were used for expression in E. coli .

Immunoprecipitation and immunoblotting

293-T cells were washed in PBS and lysed in lysis buffer (50 mM Tris pH 7.5, 0.5% NP-40, 120 mM NaCl) containing a protease inhibitor cocktail (Roche). IP was carried out by incubating 500 μg of clarified lysates with 5 μg αChk2, 1.5 μg of αPlk1, 3 μg of αHA, and 10 μl of αFlag affinity agarose gel (Sigma) at 4°C overnight. For IB, nitrocellulose membranes were blocked in 3% non-fat dry milk in TBST (0.5% Tween-20, 120mM NaCl, 50mM Tris-HCl pH 7.5) for 1h at RT, and incubated with either αPlk1 antibody (1:500), mouse αChk2 antibody (1:200), αHA-HRP antibody (1:1000) or αFlag-HRP antibody (1: 2000) overnight at 4°C. HRP-conjugated rabbit anti-mouse and goat anti-rabbit IgG (Pierce)(1:10 000) were used as secondary antibodies. IB proteins were detected by chemiluminescence reagents (Amersham Pharmacia Biotech).

Immunofluorescence microscopy

Cells grown on poly-D-lysine coated culture slides (Becton Dickinson) were washed in PBS, fixed for 15 min in PBS containing 4% paraformaldehyde and permeabilized in Triton buffer (0.5% Triton X-100 in PBS). Fixed cells were blocked in blocking solution (2% BSA, 0.1% Tween, PBS) for 30 min at 37°C in a humidified chamber. IF was performed using αPT68-Chk2 antibody (0.5 μg/ml), αPlk1 antibody (1: 100), αγ-tubulin antibody (1:1000), αPT26/PS28-Chk2 antibody (1:300), mouse αChk2

antibody (1:50), αCENP-E antibody (1:250 in 5% normal donkey serum) for 30 min at 37°C, followed by three washes in blocking buffer. Cells were incubated with anti-mouse Rhodamine (Rd) (1:1000), anti-mouse fluorescein isothiocyanate (FITC) (1:100), anti rabbit-FITC (1:100) secondary antibodies. DNA was stained with 6'-diamidino-2-phenylindole (DAPI) in mounting solution (Vector Laboratories). IF microscopy was performed by Nikon Microphot-FX microscope using 40x and 60x Plan Apo objectives. Images were taken by Spot digital camara (Diagnostic Instruments Inc.) and processed using Adobe Photoshop and Microsoft Powerpoint software.

For phosphatase treatment cells were permeabilized by treatment of cells with 0.1% Triton X-100 in PBS for 30 sec at RT, followed by three washes with PBS, once in phosphatase buffer and then incubated with 4U/μl λ-phosphatase (NEB) for 20 min at 30°C. After treatment, cells were washed in PBS and fixed as in the basic protocol.

In vitro kinase assays

Anti-Plk1 antibody (1:500) was incubated with 500 μg of lysate at 4°C overnight. Plk1 immune complexes were incubated with 10 μg α-casein at 30°C for 5 min in 20μl kinase buffer (20mM HEPES pH 7.4, 50mM KCl, 10mM MgCl2, 1mM DTT, and 1μM ATP) supplemented with 5μCi [γ-32P] ATP (Amersham Pharmacia Biotech). The reactions were stopped with Laemmli sample buffer. The samples were separated by SDS-PAGE gel, which was stained with Coomassie blue, dried, and visualized by autoradiography.

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Footnotes

Abbreviations used in this paper: ATM, Ataxia-telangiectasia mutated; DSBs, double-strand DNA breaks; GST, glutathione S-transferase; HA, hemagglutinin; IB, immunoblot; IF, immunofluoresence; IR, ionizing radiation; PIKK, phosphatidyl inositol (3') kinase-like kinase; PLKs, Polo-like kinases; SCD, SQ/TQ cluster domain.

References

Ahn, J.Y., X. Li, H.L. Davis, and C.E. Canman. 2002. Phosphorylation of Threonine 68 Promotes Oligomerization and Autophosphorylation of the Chk2 Protein Kinase via the Forkhead- associated Domain. *J Biol Chem.* 277:19389-19395.

Ahn, J.Y., J.K. Schwarz, H. Piwnica-Worms, and C.E. Canman. 2000. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Res.* 60:5934-5936.

Arnaud, L., J. Pines, and E.A. Nigg. 1998. GFP tagging reveals human Polo-like kinase 1 at the kinetochore/centromere region of mitotic chromosomes. *Chromosoma.* 107:424-429.

Bartek, J., J. Falck, and J. Lukas. 2001. CHK2 kinase--a busy messenger. *Nat Rev Mol Cell Biol.* 2:877-886.

Bell, D.W., J.M. Varley, T.E. Szydlo, D.H. Kang, D.C. Wahrer, K.E. Shannon, M. Lubratovich, S.J. Verselis, K.J. Isselbacher, J.F. Fraumeni, J.M. Birch, F.P. Li, J.E. Garber, and D.A. Haber. 1999. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science.* 286:2528-2531.

Brown, A.L., C.H. Lee, J.K. Schwarz, N. Mitiku, H. Piwnica-Worms, and J.H. Chung. 1999. A human Cds1-related kinase that functions downstream of ATM protein in the cellular response to DNA damage. *Proc Natl Acad Sci U S A.* 96:3745-3750.

Carroll, P.E., M. Okuda, H.F. Horn, P. Biddinger, P.J. Stambrook, L.L. Gleich, Y.Q. Li, P. Tarapore, and K. Fukasawa. 1999. Centrosome hyperamplification in human cancer: chromosome instability induced by p53 mutation and/or Mdm2 overexpression. *Oncogene.* 18:1935-1944.

Chaturvedi, P., W.K. Eng, Y. Zhu, M.R. Mattern, R. Mishra, M.R. Hurle, X. Zhang, R.S. Annan, Q. Lu, L.F. Faucette, G.F. Scott, X. Li, S.A. Carr, R.K. Johnson, J.D. Winkler, and B.B. Zhou. 1999. Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. *Oncogene.* 18:4047-4054.

Chehab, N.H., A. Malikzay, M. Appel, and T.D. Halazonetis. 2000. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. *Genes Dev.* 14:278-288.

Cheng, L., L. Hunke, and C.F. Hardy. 1998. Cell cycle regulation of the Saccharomyces cerevisiae polo-like kinase cdc5p. *Mol Cell Biol.* 18:7360-7370.

Descamps, S., and C. Prigent. 2001. Two mammalian mitotic aurora kinases: who's who? *Sci STKE.* 2001:E1.

Falck, J., N. Mailand, R.G. Syljuasen, J. Bartek, and J. Lukas. 2001. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature.* 410:842-847.

Fukasawa, K., T. Choi, R. Kuriyama, S. Rulong, and G.F. Vande Woude. 1996. Abnormal centrosome amplification in the absence of p53. *Science.* 271:1744-1747.

Gillett, E.S., and P.K. Sorger. 2001. Tracing the pathway of spindle assembly checkpoint signaling. *Dev Cell.* 1:162-164.

Golsteyn, R.M., K.E. Mundt, A.M. Fry, and E.A. Nigg. 1995. Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle function. *J Cell Biol.* 129:1617-1628.

Hofmann, W.K., C.W. Miller, K. Tsukasaki, S. Tavor, T. Ikezoe, D. Hoelzer, S. Takeuchi, and H.P. Koeffler. 2001. Mutation analysis of the DNA-damage checkpoint

gene CHK2 in myelodysplastic syndromes and acute myeloid leukemias. *Leuk Res.* 25:333-338.
Hsu, L.C., and R.L. White. 1998. BRCA1 is associated with the centrosome during mitosis. *Proc Natl Acad Sci U S A.* 95:12983-12988.
Hu, F., Y. Wang, D. Liu, Y. Li, J. Qin, and S.J. Elledge. 2001. Regulation of the Bub2/Bfa1 GAP complex by Cdc5 and cell cycle checkpoints. *Cell.* 107:655-665.
Ingvarsson, S., B.I. Sigbjornsdottir, C. Huiping, S.H. Hafsteinsdottir, G. Ragnarsson, R.B. Barkardottir, A. Arason, V. Egilsson, and J.T. Bergthorsson. 2002. Mutation analysis of the CHK2 gene in breast carcinoma and other cancers. *Breast Cancer Res.* 4.
Jullien, D., P. Vagnarelli, W.C. Earnshaw, and Y. Adachi. 2002. Kinetochore localisation of the DNA damage response component 53BP1 during mitosis. *J Cell Sci.* 115:71-79.
Lange, B.M. 2002. Integration of the centrosome in cell cycle control, stress response and signal transduction pathways. *Curr Opin Cell Biol.* 14:35-43.
Lee, J.S., K.M. Collins, A.L. Brown, C.H. Lee, and J.H. Chung. 2000. hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response. *Nature.* 404:201-204.
Mantel, C., S.E. Braun, S. Reid, O. Henegariu, L. Liu, G. Hangoc, and H.E. Broxmeyer. 1999. p21(cip-1/waf-1) deficiency causes deformed nuclear architecture, centriole overduplication, polyploidy, and relaxed microtubule damage checkpoints in human hematopoietic cells. *Blood.* 93:1390-1398.
Matsuoka, S., M. Huang, and S.J. Elledge. 1998. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science.* 282:1893-1897.
Matsuoka, S., T. Nakagawa, A. Masuda, N. Haruki, S.J. Elledge, and T. Takahashi. 2001. Reduced expression and impaired kinase activity of a Chk2 mutant identified in human lung cancer. *Cancer Res.* 61:5362-5365.
Matsuoka, S., G. Rotman, A. Ogawa, Y. Shiloh, K. Tamai, and S.J. Elledge. 2000. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proc Natl Acad Sci U S A.* 97:10389-10394.
Melchionna, R., X.B. Chen, A. Blasina, and C.H. McGowan. 2000. Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. *Nat Cell Biol.* 2:762-765.
Meraldi, P., R. Honda, and E.A. Nigg. 2002. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53-/- cells. *Embo J.* 21:483-492.
Miller, C.W., T. Ikezoe, U. Krug, W.K. Hofmann, S. Tavor, V. Vegesna, K. Tsukasaki, S. Takeuchi, and H.P. Koeffler. 2002. Mutations of the CHK2 gene are found in some osteosarcomas, but are rare in breast, lung, and ovarian tumors. *Genes Chromosomes Cancer.* 33:17-21.
Nigg, E.A. 1998. Polo-like kinases: positive regulators of cell division from start to finish. *Curr Opin Cell Biol.* 10:776-783.
Pellicioli, A., S.E. Lee, C. Lucca, M. Foiani, and J.E. Haber. 2001. Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. *Mol Cell.* 7:293-300.
Sanchez, Y., J. Bachant, H. Wang, F. Hu, D. Liu, M. Tetzlaff, and S.J. Elledge. 1999. Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. *Science.* 286:1166-1171.
Schiebel, E. 2000. gamma-tubulin complexes: binding to the centrosome, regulation and

microtubule nucleation. *Curr Opin Cell Biol.* 12:113-118.
Shieh, S.Y., J. Ahn, K. Tamai, Y. Taya, and C. Prives. 2000. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. *Genes Dev.* 14:289-300.
Skibbens, R.V., and P. Hieter. 1998. Kinetochores and the checkpoint mechanism that monitors for defects in the chromosome segregation machinery. *Annu Rev Genet.* 32:307-337.
Smith, L., S.J. Liu, L. Goodrich, D. Jacobson, C. Degnin, N. Bentley, A. Carr, G. Flaggs, K. Keegan, M. Hoekstra, and M.J. Thayer. 1998. Duplication of ATR inhibits MyoD, induces aneuploidy and eliminates radiation-induced G1 arrest. *Nat Genet.* 19:39-46.
Smits, V.A., R. Klompmaker, L. Arnaud, G. Rijksen, E.A. Nigg, and R.H. Medema. 2000. Polo-like kinase-1 is a target of the DNA damage checkpoint. *Nat Cell Biol.* 2:672-676.
Toczyski, D.P., D.J. Galgoczy, and L.H. Hartwell. 1997. CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. *Cell.* 90:1097-1106.
Tutt, A., A. Gabriel, D. Bertwistle, F. Connor, H. Paterson, J. Peacock, G. Ross, and A. Ashworth. 1999. Absence of Brca2 causes genome instability by chromosome breakage and loss associated with centrosome amplification. *Curr Biol.* 9:1107-1110.
van Vugt, M.A., V.A. Smits, R. Klompmaker, and R.H. Medema. 2001. Inhibition of Polo-like kinase-1 by DNA damage occurs in an ATM- or ATR- dependent fashion. *J Biol Chem.* 276:41656-41660.
Ward, I.M., X. Wu, and J. Chen. 2001. Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. *J Biol Chem.* 276:47755-47758.
Xu, X., L.M. Tsvetkov, and D.F. Stern. 2002. Chk2 activation and phosphorylation-dependent oligomerization. *Mol Cell Biol.* 22:4419-4432.
Zhou, B.B., P. Chaturvedi, K. Spring, S.P. Scott, R.A. Johanson, R. Mishra, M.R. Mattern, J.D. Winkler, and K.K. Khanna. 2000. Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. *J Biol Chem.* 275:10342-10348.

Figure legends

Figure 1. Characterization of αPT68-Chk2 antibody. (A) Whole cell extracts from HEK 293-T cells transiently expressing HA-Chk2-wild type (lanes 1 and 2) or HA-Chk2T68A (lanes 3 and 4) and mock-irradiated (lanes 1 and 3) or irradiated with 5 Gy (lanes 2 and 4) IR were IB with αPT68-Chk2, αChk2, or αHA antibodies. (B) IB of whole cell extracts from HEK 293-T cells exposed to increasing doses of IR (0, 2, 5, 10, and 20 Gy) detected using αPT68-Chk2, or αChk2 antibodies. (C) IB of purified recombinant GST-Chk2-WT and kinase-defective GST-Chk2 D368A fusion proteins, produced in bacteria, developed with αPT68-Chk2 or αChk2 antibodies. (D) IF of non-irradiated (0 Gy) and irradiated (4 Gy) HEK-293, HT-1080, and GM5849C cells with αPT68-Chk2 antibody. Bars are 10 μm.

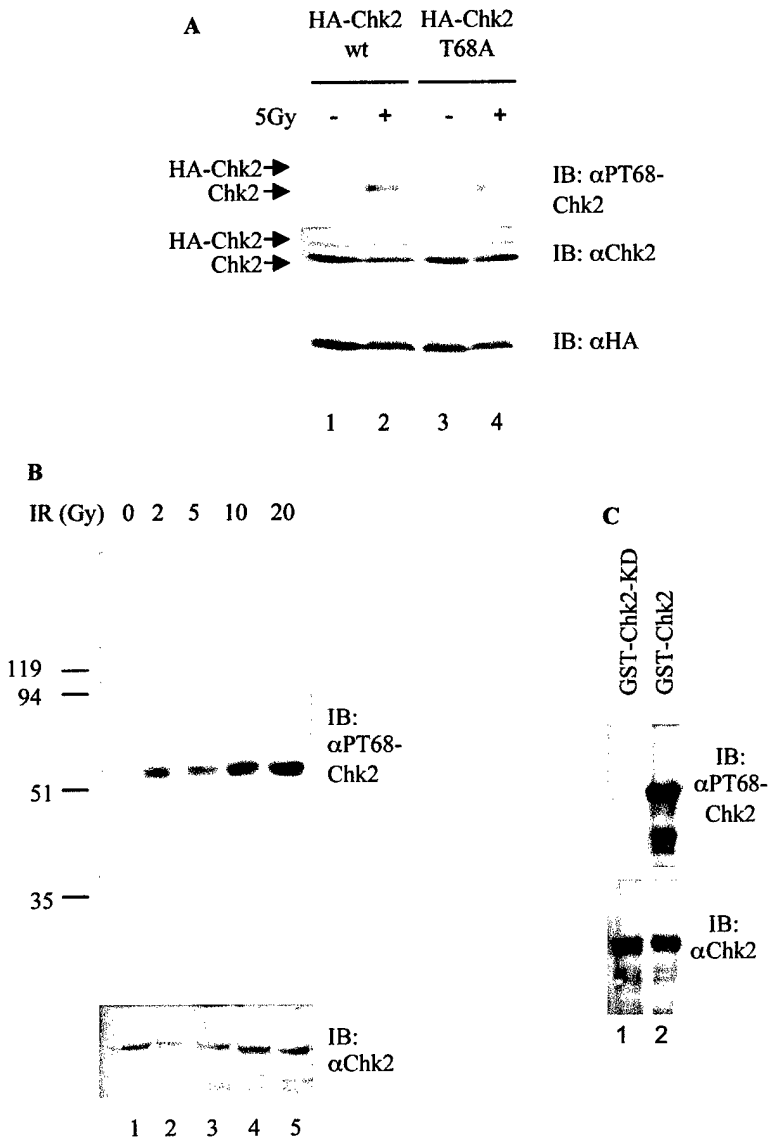
Figure 2. Centrosomal localization of phosphorylated Chk2. (A) IF of HEK-293 cells with αPT68-Chk2 antibody detected with FITC. DAPI staining marks cell nuclei. (B) Co-immunostaining of U2-OS cells with αPT68-Chk2 (marked with FITC) and αγ-tubulin (marked with rhodamine) antibodies. (C) Phosphopeptide immunogen used to raise αPT68-Chk2 antibody was used for competition with the antigen in immunostaining of U2-OS cells with αPT68-Chk2 and αγ-tubulin antibodies. (D) U2-OS cells were co-immunostained with αPT68-Chk2 and αγ-tubulin antibodies after *in situ* permeabilization and treatment with 4U/μl λ-phosphatase or buffer for 30 min at 25^0C. (E) Co-immunostaining of U2-OS cells with αPT68-Chk2 and αChk2 antibodies. Arrowheads mark coincident staining between αPT68-Chk2 and a subset the αChk2 signal. Bars are 10 μm.

Figure 3. Localization of phosphorylated Chk2 to kinetochores and midbody. (A) Localization of phosphorylated Chk2 to kinetochores detected by IF of U2-OS cells with αPT26/PS28-Chk2 antibody (green), DAPI staining for DNA (blue). (B) Midbody localization of phosphorylated Chk2. IF of U2-OS cells with αPT68-Chk2 (red) and αPT26/PS28-Chk2 (green) antibodies, DAPI (blue). (C) Co-localization of phosphorylated Chk2 and CENP-E to kinetochores. Co-immunostaining of U2-OS cells with αPT26/PS28-Chk2 (red), αCENP-E (green) antibodies, and DAPI (blue). Bars are 10 μm.

Figure 4. Chk2 interacts with Plk1. (A) Co-immunoprecipitation of HA-Chk2 and Flag-Plk1. Whole cell extracts from HEK 293-T cells transiently expressing HA-Chk2 (lanes 1, 2, 3, 5, and 6) and/or Flag-Plk1 (lanes 2, 3, 4, 5, and 6) were immunoprecipitated with mouse IgG (lanes 2 and 5), αFlag antibody (lanes 1 and 3), or αHA antibody (lanes 4 and 6). IB analysis of immunoprecipitates was performed with αFlag (lower panel) and αHA antibodies (upper panel). (B) Co-IP of endogenous Chk2 and Plk1. HEK 293-T cells were treated with 0 (lanes 1, 2, 7, and 8) or 250 ng/ml (lanes 3, 4, 5, 6, 9, and 10) nocodazole for 16 hours and with 0 (lanes 1, 3, 5, 6, 7, and 9) or 0.5μM (lanes 2, 4, 8, and 10) adriamycin for 1 h. Whole cell extracts from the cells were IP with pre-immune serum (lane 5), mouse IgG (lane 6), αChk2 antibody (lanes 1-4) and αPlk1 antibody (lanes 7-10). IB analyses were performed to detect Chk2 (lower left panel) and Plk1 (upper left and upper right panels) in the immunoprecipitates. A portion of Plk1

immunoprecipitates were incubated with [γ-32P]ATP for *in vitro* kinase assay. Kinase activity of Plk1 was detected by incorporation of [γ-32P]ATP into α-casein, used as a substrate (middle right). Amounts of Plk1 used in *in vitro* kinase assay were monitored by IB analysis of Plk1 immunoprecipitates (upper right). α-casein was detected by Coomassie brilliant blue staining (lower right).

Figure 5. Co-localization of Chk2 and Plk1 to the centrosome and the midbody. Co-immunostaining of U2-OS cells with αPT68-Chk2 (green) and αPlk1 (red) antibodies. Signals of αPT68-Chk2 and αPlk1 antibodies overlap at the centrosome in prophase (A) and metaphase (B) cells and at the midbody later in mitosis (C). DAPI staining for the location of DNA. Bars are 10 μm.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HA-Chk2 T68A
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HA-Chk2
Chk2
IB: αPT68-Chk2
HA-Chk2
Chk2
IB: αChk2
IB: αHA
1 2 3 4
B
IR (Gy) 0 2 5 10 20
119
94
51
35
IB: αPT68-Chk2
IB: αChk2
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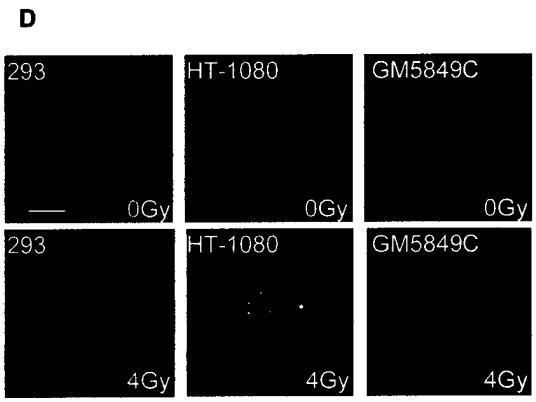
1 2

Figure 1


D
293
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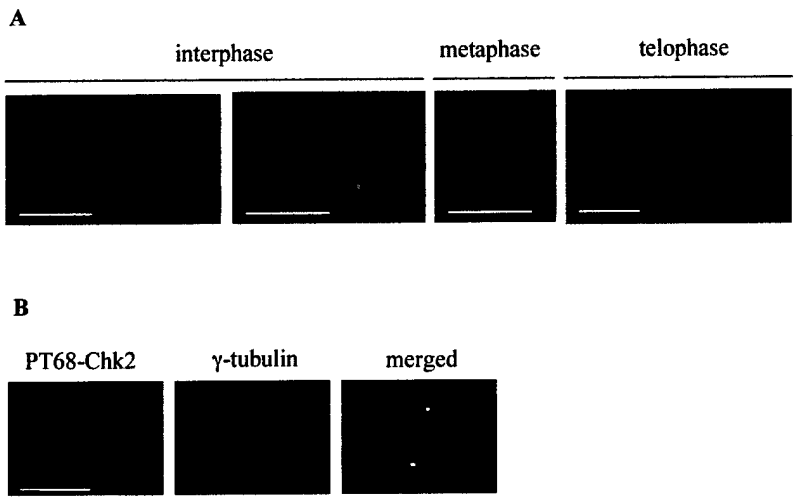
HT-1080
0Gy
GM5849C
0Gy
293
4Gy
HT-1080
4Gy
GM5849C
4Gy

Figure 1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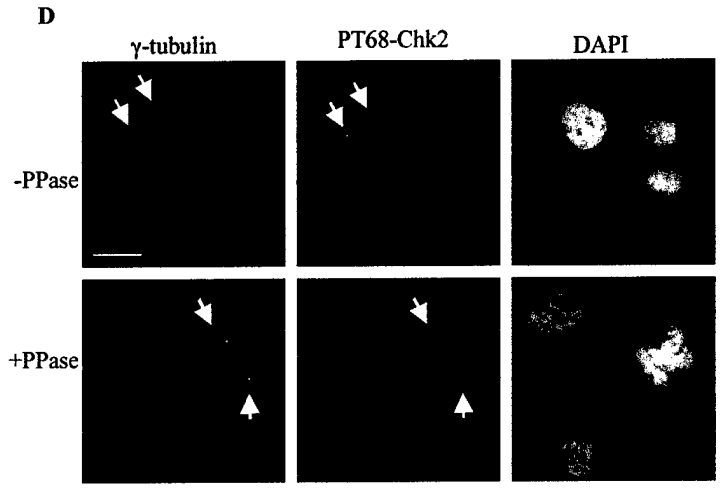
interphase
metaphase
telophase
B
PT68-Chk2
γ-tubulin
merged
C
γ-tubulin
PT68-Chk2
DAPI
-peptide
+peptide

Figure 2



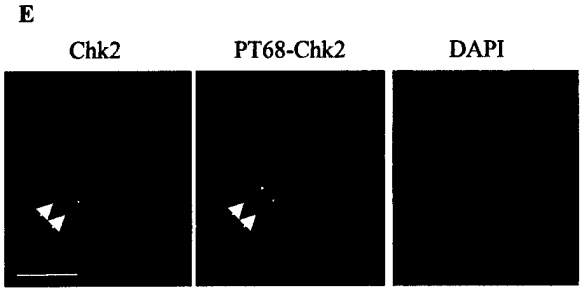


Figure 2

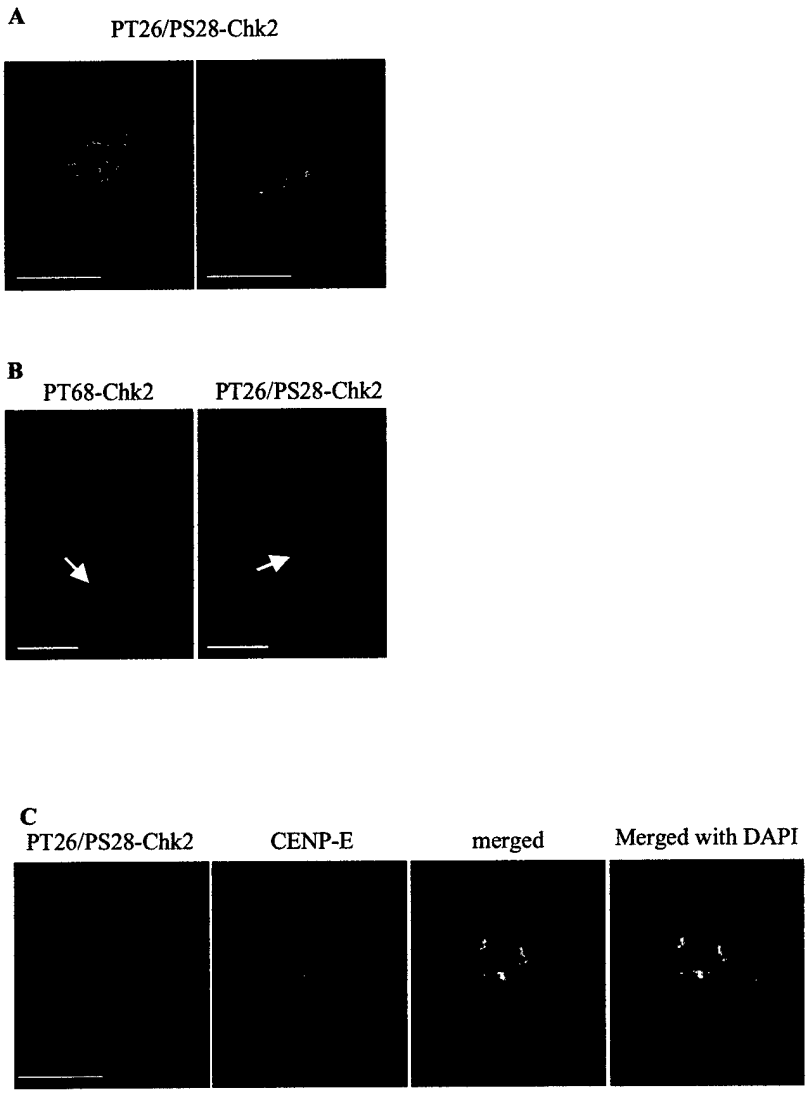
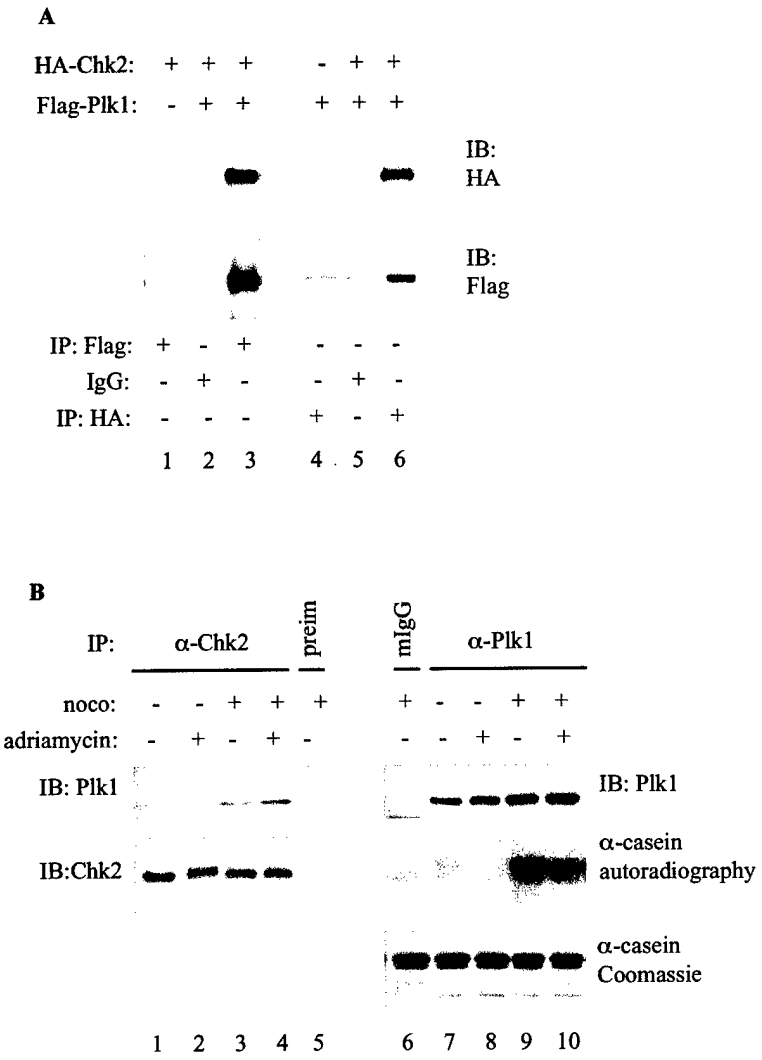

A
PT26/PS28-Chk2
B
PT68-Chk2
PT26/PS28-Chk2
C
PT26/PS28-Chk2
CENP-E
merged
Merged with DAPI

Figure 3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Flag-Plk1: - + + + + +
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IB: Flag
IP: Flag: + - + - - -
IgG: - + - - + -
IP: HA: - - - + - +
1 2 3 4 5 6
B
IP: α-Chk2 preim mIgG α-Plk1
noco: - - + + + + - - + +
adriamycin: - + - + - - - + - +
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IB:Chk2
IB: Plk1
α-casein autoradiography
α-casein Coomassie
1 2 3 4 5 6 7 8 9 10

Figure 4

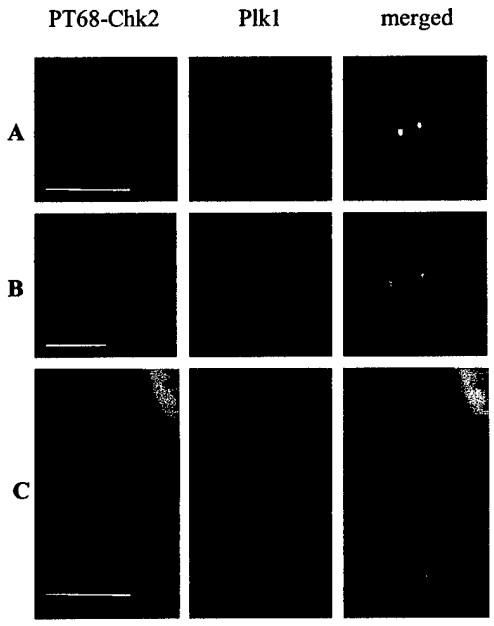

Figure 5

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Chk2 Activation and Phosphorylation-Dependent Oligomerization

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The tumor suppressor gene *CHK2* encodes a versatile effector serine/threonine kinase involved in responses to DNA damage. Chk2 has an amino-terminal SQ/TQ cluster domain (SCD), followed by a forkhead-associated (FHA) domain and a carboxyl-terminal kinase catalytic domain. Mutations in the SCD or FHA domain impair Chk2 checkpoint function. We show here that autophosphorylation of Chk2 produced in a cell-free system requires *trans* phosphorylation by a wortmannin-sensitive kinase, probably ATM or ATR. Both SQ/TQ sites and non-SQ/TQ sites within the Chk2 SCD can be phosphorylated by active Chk2. Amino acid substitutions in the SCD and the FHA domain impair auto- and *trans*-kinase activities of Chk2. Chk2 forms oligomers that minimally require the FHA domain of one Chk2 molecule and the SCD within another Chk2 molecule. Chk2 oligomerization in vivo increases after DNA damage, and when damage is induced by gamma irradiation, this increase requires *ATM*. Chk2 oligomerization is phosphorylation dependent and can occur in the absence of other eukaryotic proteins. Chk2 can cross-phosphorylate another Chk2 molecule in an oligomeric complex. Induced oligomerization of a Chk2 chimera in vivo concomitant with limited DNA damage augments Chk2 kinase activity. These results suggest that Chk2 oligomerization regulates Chk2 activation, signal amplification, and transduction in DNA damage checkpoint pathways.

DNA damage activates response pathways that halt the cell cycle, induce the transcription of genes that facilitate DNA repair and DNA replication, alter telomeres, and induce apoptosis if damage cannot be repaired (65). Checkpoint defects may result in genomic instability, a mutagenic condition that predisposes organisms to cancer. On the other hand, DNA-damaging agents, in the form of gamma irradiation and genotoxic drugs, are mainstays of current cancer treatment regimens. Manipulation of checkpoint genes may ultimately benefit chemo- and radiotherapy (23).

Checkpoint pathways are analogous to growth factor-regulated signal transduction pathways, in which DNA damage initiates a signal that is transduced and amplified to generate checkpoint responses. Although the precise nature of the initial step of signal transduction is poorly understood, damaged DNA activates a cascade of protein kinases. In mammals, these kinases include the phosphoinositide kinase-related kinases (PIKKs) Atm (ataxia telangiectasia mutated) and Atr (Atm and Rad3 related) and the downstream serine/threonine checkpoint kinases Chk1 and Chk2. Orthologs of these genes have been identified in yeasts, with *Saccharomyces cerevisiae* Mec1 and Tel1 serving Atm- or Atr-like functions and Chk1 and Rad53 resembling mammalian Chk1 and Chk2, respectively. Effectors that execute the functions of the DNA damage responses include substrates of both PIKKs and checkpoint kinases.

Atm is a central signaling protein in the response to ionizing radiation and other sources of double-strand DNA breaks. Homozygous mutations in *ATM* are responsible for the pleiotropic ataxia-telangiectasia syndrome, which includes cancer predisposition and sensitivity to ionizing radiation along with progressive cerebellar defects (29). Chk2 is a major effector of Atm (6, 7, 10, 40). Both the breast cancer susceptibility gene product Brca1 (16, 33) and p53 (3, 8, 11, 12, 25, 52) are substrates of Atm and Chk2. Li-Fraumeni syndrome is a hereditary disorder predisposing to multiple neoplasms and is generally associated with a constitutional *TP53* mutation. *CHK2* mutations have been identified in some Li-Fraumeni syndrome kindreds that do not have p53 mutations (5, 58), in myelodysplastic syndromes and acute myeloid leukemias (27), in lung cancer (24, 44), in osteosarcoma (44), and in ovarian cancer (44). *CHK2*, therefore, is a regulator of tumor suppressor gene products and is itself a likely tumor suppressor gene.

Chk2 activation in response to ionizing irradiation is *ATM* dependent (6, 7, 10, 40). Activated Chk2 in turn phosphorylates p53 at serine-20 (11, 25, 52), Cdc25A at serine-123 (21), and Cdc25C at serine-216 (6, 7, 10, 40), contributing to the G_1/S, S, and G_2/M checkpoints, respectively.

Atm-like PIKKs show a strong sequence preference for phosphorylation of SQ/TQ sites (2, 30). The SQ/TQ cluster domain (SCD), near the amino terminus of Chk2, includes seven SQ/TQ sites, including known *ATM*- and *ATR*-dependent phosphorylation targets (1, 41, 42, 64). The SCD is followed by a forkhead-associated (FHA) domain and a carboxyl-terminal kinase catalytic domain. Activation of Chk2 occurs through a series of steps, including *trans* phosphorylation by Atm or Atr at sites within the SCD, including T68 (1, 41, 42, 64). PIKK-dependent phosphorylation is required for autophosphorylation within the activation loop of the catalytic domain at T383 and/or T387 (32).

The intact FHA domain is required for damage-dependent activation of Chk2 (11, 32). FHA protein homology domains were first identified in a subset of forkhead transcription factors (26). They are present in a wide variety of proteins in prokaryotes and eukaryotes (34). Many eukaryotic FHA domain-containing proteins are found in the nucleus and are involved in DNA repair and cell cycle arrest (34). Recent work

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shows that FHA domains are phosphopeptide recognition domains (18, 19, 36, 37, 55, 57), and structurally they have some similarity to the MH2 domains of R-Smads (19), signal transducers and transcriptional comodulators of transforming growth factor beta (TGF-β) signaling (39). However, only a small number of protein-protein interactions that are mediated by FHA domains have been identified. They include association of an *Arabidopsis thaliana* phosphatase FHA domain with a phosphorylated receptor-like kinase (35, 55) and interaction of *S. cerevisiae* Rad53 with phosphorylated Rad9 (57), which operates upstream from Rad53 in the damage-dependent signaling cascade.

The FHA domains of the *S. cerevisiae* Chk2 homolog Rad53 couple Rad53 to damage-dependent signals through direct binding to a second damage response protein, Rad9 (57). DNA damage induces PIKK-dependent phosphorylation of Rad9 (20, 47, 56, 59). Once phosphorylated, Rad9 binds tightly to the two FHA domains of Rad53 (18, 36, 37, 57). Disruption of this interaction either through mutations of Rad53 FHA domains (57) or mutations in the Rad9 sites that bind Rad53 FHA domains (49a) prevents activation of Rad53. Since Rad9 and Rad53 both require Mec1 for activating phosphorylations, these results suggested that phospho-Rad9 acts as an adaptor that recruits Rad53 to an activating complex containing Mec1. Alternatively, it has been proposed that phosphorylated Rad9 dimer functions as a scaffold to bring Rad53 molecules into close proximity to each other, facilitating cross-phosphorylation between Rad53 molecules and subsequent release of activated Rad53 (22).

Activation of protein kinases through regulated oligomerization has been demonstrated for both tyrosine kinases and serine/threonine kinases (28, 38, 50). We report here that Chk2 undergoes oligomerization in response to DNA damage. This process is mediated by the phosphorylated SCD in an activated Chk2 molecule and the FHA domain in another Chk2 molecule. With limited DNA damage, oligomerization of Chk2 modulates phosphorylation and kinase activity. We propose that Chk2 oligomerization is central to regulation of Chk2 activation, signal transduction, and signal amplification.

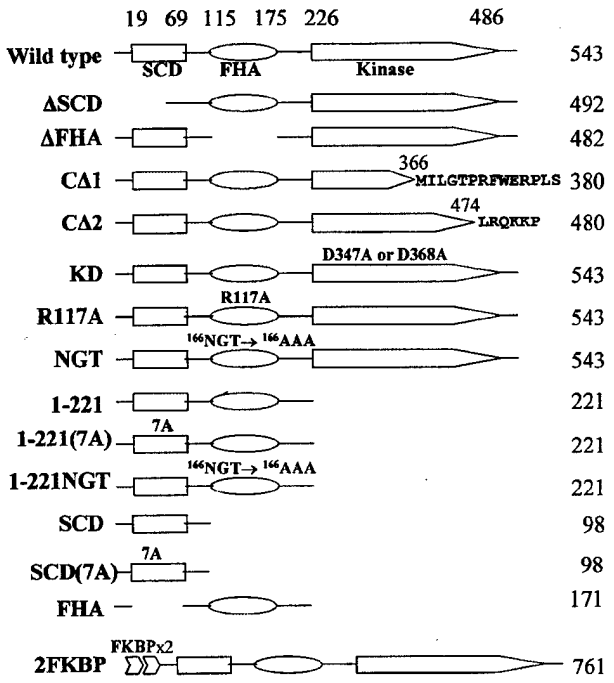


FIG. 1. Schematic diagrams of Chk2 and Chk2 mutants. SCD, FHA, and kinase catalytic domains are marked, with amino acid coordinates above. CΔ1 corresponds to a spontaneous variant of *CHK2* from Li-Fraumeni syndrome, with a frameshift mutation causing readthrough into alternate reading frames as indicated. CΔ2, a similar frameshift mutation thought to be a spontaneous variant of *CHK2* from Li-Fraumeni syndrome, was found to be a polymorphism in the homologous fragment present on chromosome 15 (53). KD is kinase defective owing to substitutions of conserved residues in the catalytic domain. R117A and NGT/AAA are substitutions of conserved FHA domain residues. 7A has alanine substituted for each S or T of all seven SQ and TQ sites within the SCD. 2FKBP contains two copies of the FKBP mutant (F36V) fused to the amino terminus of Chk2.

MATERIALS AND METHODS

Antibodies. Rabbit polyclonal anti-Chk2 T26/S28 was a kind gift from Yi Tan (Cell Signaling Technology). This antibody recognizes Chk2 after gamma irradiation, but not when T26 and S28 have been replaced with alanine (X. Xu and D. F. Stern, unpublished data). Rabbit anti-phospho-T68 was produced by immunization with keyhole limpet hemocyanin coupled to KSSLETVSpTQELYSI, where pT is phosphothreonine. This antibody recognizes Chk2 after gamma irradiation, but not when T68 has been replaced with alanine (Fig. 7E and data not shown). Mouse monoclonal anti-HA antibody (16B12) was purchased from Covance; mouse monoclonal anti-Flag, rabbit anti-glutathione-*S*-transferase (GST), and mouse immunoglobulin G (IgG) were purchased from Sigma; horseradish peroxidase-conjugated mouse antihemagglutinin (HA) (12CA5) and rat anti-HA (3F10) monoclonal antibodies were from Roche; and goat anti-Chk2 (N-17) was from Santa Cruz. Antigen-antibody complexes were recovered with protein G plus protein A-agarose (CalBiochem). Horseradish peroxidase-conjugated secondary antibodies and chemiluminescence reagents were from Pierce.

Plasmids (Fig. 1). A clone within the expressed sequence tag database (GenBank accession no. AA285249) containing the entire coding sequence of Chk2 was obtained from Thanos D. Halazonetis (Wistar Institute). For expression in mammalian cells, Chk2-coding sequences were amplified by PCR and cloned into the pcDNA3xHA-Neo and pcDNA3xFlag-Neo vectors, resulting in pcDNA-HAChk2 and pcDNA-FlagChk2, respectively. Point and internal deletion mutants were generated from pcDNA-HAChk2 by PCR-based site-directed mutagenesis (63).

For expression in *Escherichia coli*, Chk2 sequences (Fig. 1) were cloned into pGEX4T vectors (Amersham Pharmacia Biotech) for GST fusions and pTrcHis vectors (Invitrogen) for His-tagged fusions. Human Cdc25C (amino acids 200 to 256) and Cdc25A (amino acids 101 to 140) were isolated by PCR from expressed sequence tag clones (GenBank accession no. AW401554 and BE743496, respectively). Two copies of the FK506-binding protein (FKBP) mutant were isolated by PCR from pC4M-Fv2E (Ariad Pharmaceuticals, Inc., Cambridge, Mass.), digested with *Apo*I and *Eco*RI, and subcloned into the *Eco*RI site of the pcDNA3xHA and pcDNA3xFlag vectors. The resulting constructs were designated pcDNAHA2FKBP and pcDNAFlag2FKBP, respectively. Chk2 and its mutants were subcloned into these vectors.

Plasmid constructs were verified by sequence analysis. Primer sequences and detailed cloning strategies are available upon request. Wild-type and kinase-defective (D2870A and N2875K, respectively) Flag-*ATM* constructs (8) were kind gifts from Michael Kastan (St. Jude Children's Hospital). pGEX-Chk2(1-222) and pGEX-Chk2(57-222) (9) were obtained from Susan Lees-Miller (University of Calgary). pC4M-Fv2E, containing two copies of mutated FKBP, was provided by Ariad Pharmaceuticals, Inc. (Cambridge, Mass.).

Recombinant proteins. Expression of GST fusions or His fusions in *E. coli* strain DH5α was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 to 4 h at 37°C. For GST fusion proteins, cell lysates were harvested in PBS (Dulbecco's phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}) in the presence of 1 mg of lysozyme and 10 U of DNase I per ml. For His fusion proteins, cell lysates were collected in 50 mM NaH_2PO_4–300 mM NaCl–10 mM imidazole, pH 8.0, in the presence of 1 mg of lysozyme and 10 U of DNase I per

ml. Cell lysates then went through 10 cycles of freezing and thawing. GST and His fusion proteins were batch purified with glutathione-Sepharose beads (Amersham Pharmacia Biotech) or Ni-nitrilotriacetic acid (NTA) beads (Qiagen), respectively, according to the manufacturers' procedures. GST fusion proteins were eluted with 50 mM Tris–10 mM glutathione, pH 8.0. His fusion proteins were eluted with 50 mM NaH_2PO_4–300 mM NaCl–250 mM imidazole, pH 8.0, and then dialyzed against PBS.

In vitro coupled transcription-translation assays. Chk2 constructs (pcDNA series) were used as templates for in vitro transcription-translation of Chk2 in the absence or presence of [^{35}S]Met-Cys labeling mix (Amersham Pharmacia Biotech). Promega TNT T7 quick coupled transcription-translation reticulocyte lysate system and T7 coupled transcription-translation wheat germ extract system were used in a standard 50-μl reaction according to procedures recommended by the manufacturer.

Cell culture and transfection. *ATM*-deficient (GM5849C) simian virus 40-transformed human fibroblasts were obtained from the Coriell Institute for Medical Research, Camden, N.J. Other cell lines were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U of penicillin/ml, and 50 mg of streptomycin/ml. Transfection was performed with Fugene 6 (Roche) at a ratio of 1 μg of plasmid to 2 μl of Fugene. Stable transfectants were selected in medium containing G418 (Life Technologies) at 700 μg/ml. Cells were treated with 1 mM hydroxyurea (Sigma) 24 h after transfection for 24 h. Cells were irradiated in a Mark I ^{137}Cs irradiator (Shepherd) at a dose rate of 1.8 Gy/min 48 h after transfection. Cells were UV irradiated at a dose of 50 J/m^2 with a Stratagene Cross-linker 48 h after transfection.

Immunoprecipitation and immunoblotting. Cell lysate was harvested 2 h after irradiation or 24 h after hydroxyurea treatment in TSD buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 0.1% sodium deoxycholate, 0.1% Triton X-100, and protease inhibitor cocktail [Roche]). Two micrograms of antibodies was used for immunoprecipitation from 400 to 500 μg of total lysate at 4°C overnight. Precipitates were washed with TSD buffer lacking protease inhibitors. In vitro translation product was mixed with 300 μl of NETN (20 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail) for immunoprecipitation. Precipitates were washed with NETN buffer lacking protease inhibitors. Immunoblots on nitrocellulose were blocked with 5% nonfat milk in PBST (PBS with 0.5% Tween 20) and washed in PBST.

GST pulldown experiments. Two micrograms of soluble GST fusion proteins and 20 μl of glutathione-Sepharose beads were incubated with 500 μg of total lysate in TSD buffer derived from HEK 293 cells expressing HA-tagged Chk2 and mutants (Fig. 6C, 7C, and 7D) or with 0.5 μg of soluble wild-type or kinase-defective His-Flag-Chk2 in the presence of 300 μl of NETN buffer (Fig. 6A, 6B, and 8) at 4°C overnight. The beads were washed in NETN buffer lacking protease inhibitors.

In vitro kinase assays. Kinases (prepared as soluble GST or His fusion proteins, immune complexes, or GST affinity isolates) were incubated with substrates at 30°C for 5 to 10 min in 1× kinase buffer (20 mM Tris [pH 7.5], 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol) supplemented with either 2 μM nonradioactive ATP or 2 μM nonlabeled ATP and 10 μCi of [γ-^{32}P]ATP (>5,000 Ci/mmol; AA0018, Amersham Pharmacia Biotech).

Phosphatase treatment. Immunoprecipitates of HA-Chk2 produced by translation in the coupled reticulocyte lysate system were incubated with calf intestinal phosphatase (New England Biolabs) for 1 h at 37°C. Soluble wild-type His-Flag-Chk2 (0.5 μg) was incubated with 1,000 U of λ phosphatase (New England Biolabs) in the presence of 2 mM $MnCl_2$ for 1 to 2 h at 30°C in a 50-μl reaction volume.

Induced oligomerization of Chk2 chimeras. HEK 293 cells were transiently transfected with pcDNAHA2FKBP-Chk2 (or its kinase-defective mutant) and/or pcDNAFlag2FKBP-Chk2 (or its kinase-defective mutant). Forty-eight hours later, transfectants were either mock-treated with 0.1% ethanol or treated with a 10 nM concentration of the bivalent ligand AP20187 (Ariad Pharmaceuticals, Inc.) for the FKBP mutant. Where indicated, transfectants were exposed to 10-Gy or 2.5-Gy gamma irradiation immediately before adding ligand. Lysates were harvested between 2 and 6 h after treatment. All solutions for immunoprecipitation, washing, and kinase assays for the AP20187-treated samples contained 10 μM AP20187.

RESULTS

Cell-free system for activation of Chk2. In *S. cerevisiae*, DNA damage induces a stable modification of the Chk2 homolog Rad53 that results in elevated activity detectable by in-gel kinase assays after denaturing gel electrophoresis and subsequent renaturation (45). This modification, probably phosphorylation, depends upon the Atm/Atr homolog Mec1. However, efforts to activate mammalian Chk2 in vitro by phosphorylation with Atm (41) or DNA-dependent protein kinase (DNA-PK) (X. Xu and D. F. Stern, unpublished data) have been unsuccessful.

Chk2 produced by coupled in vitro transcription-translation in rabbit reticulocyte lysates migrates heterogeneously in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Fig. 2A, lane 1). The slower-migrating form was eliminated by phosphatase treatment (Fig. 2A, lanes 3 and 4). Expression of the kinase-defective D347A allele in rabbit reticulocyte lysates yielded only the hypophosphorylated form (Fig. 2A, lane 2), indicating that autophosphorylation is required for the mobility shift. Phosphorylation of immunoreactive T26/S28 and T68 in the SCD was evident in both wild-type and kinase-defective Chk2 derived from rabbit reticulocyte lysates (Fig. 2B, lane 12). Thus, one or more protein kinases that can phosphorylate Chk2 at these sites are present in the rabbit reticulocyte lysates. Since the kinase-defective form does not undergo an extreme mobility shift (Fig. 2A, lane 2), phosphorylation at T26/S28 and/or T68 is not sufficient to retard the mobility of Chk2.

The simplest explanation for these results is that an endogenous Chk2 activating kinase is present in this system, with likely candidates being endogenous PIKKs and/or Chk2 (see below). Preincubation of rabbit reticulocyte lysates with 1 mM caffeine or 5 μM wortmannin inhibited phosphorylation at T26/S28 and at T68 (Fig. 2B, lanes 3 and 7). At these concentrations, caffeine inhibits human Atm and, partially, Atr but not DNA-PK, and wortmannin inhibits both Atm and DNA-PK but not Atr (48, 49). Loss of phosphorylation at these sites was accompanied by loss of Chk2 autophosphorylation activity, assayed by ^{32}P incorporation (Fig. 2B, lanes 3 and 7). We conclude that Chk2 derived from the rabbit reticulocyte lysates is phosphorylated by an Atm-like kinase and that this phosphorylation is required for strong Chk2 kinase activity.

In contrast to material produced in rabbit reticulocyte lysates, HA-Chk2 derived by translation in wheat germ extracts was not hyperphosphorylated, lacked T26/S28 and T68 phosphorylation, and had minimal autophosphorylation activity (Fig. 2B, lane 14, and Fig. 2C, lane 2). This system probably lacks an endogenous kinase that is capable of activating mammalian Chk2. Incubation of this material in rabbit reticulocyte lysates enhanced T68 phosphorylation and Chk2 autophosphorylation (Fig. 2C, lane 3; compare to Chk2 produced in rabbit reticulocyte lysates in lane 5). To our knowledge, this is the first mammalian cell-free system to enable catalytic activation of Chk2.

SCD is phosphorylated by Chk2. In the rabbit reticulocyte lysate system and in intact cells exposed to ionizing radiation, kinase-defective Chk2 is phosphorylated in *trans* at T26/S28 and T68 (Fig. 2B and data not shown). However, the full phosphorylation of Chk2 resulting in electrophoretic mobility shift requires a functional Chk2 kinase domain (Fig. 2B, lanes 2 and 12, and data not shown). This suggests that, at minimum, Chk2 electrophoretic mobility shift requires successive *trans* phosphorylation and autophosphorylation. Phosphorylation of Chk2 within the activation loop of the kinase domain is re-

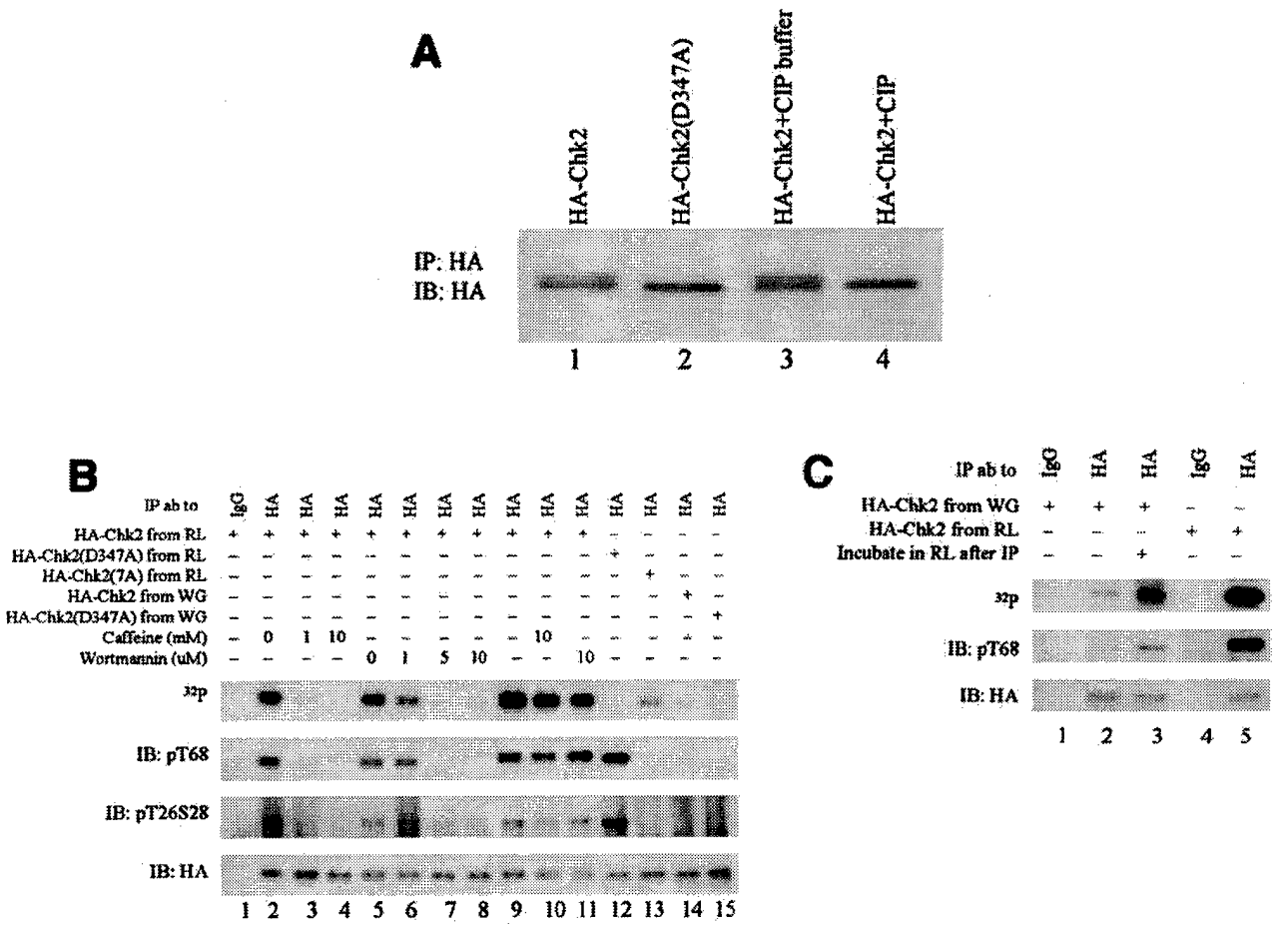


FIG. 2. Cell-free phosphorylation and activation of Chk2. HA-Chk2 or kinase-defective HA-Chk2(D347A) was produced by coupled transcription-translation in reticulocyte lysate (RL) or wheat germ lysate (WG) and isolated by immunoprecipitation with anti-HA. (A) Phosphorylation of HA-Chk2 produced in vitro. HA-Chk2 or HA-Chk2(D347A) produced in rabbit reticulocyte lysates was immunoprecipitated and detected by immunoblotting with anti-HA. For phosphatase experiments (right lanes), immunoprecipitated HA-Chk2 was incubated in calf intestinal phosphatase (CIP) buffer or calf intestinal phosphatase buffer plus calf intestinal phosphatase (1). HA-Chk2 produced in the reticulocyte lysate in vitro translation system is phosphorylated. Both HA-Chk2 and HA-Chk2(D347A) were in vitro transcribed-translated in the reticulocyte lysate with [^{35}S]Met-Cys for labeling and precipitated with anti-HA antibody. The immunoprecipitates of HA-Chk2 were treated with alkaline phosphatase buffer alone or plus alkaline phosphatase at 37°C for 1 h. IP, immunoprecipitation; IB, immunoblotting; ab, antibody. (B) Chk2 phosphorylation and kinase activity. Forms of HA-Chk2 produced by cell-free transcription-translation were immunoprecipitated and incubated with [γ-^{32}P]ATP for in vitro kinase assays. Recovery of Chk2 was monitored by immunoblotting with anti-HA (bottom). Phosphorylation at T68 or T26/S28 was measured by immunoblotting with the appropriate phosphospecific antibody; in vitro kinase activity was monitored by incorporation of [γ-^{32}P]ATP (top). The ^{32}P, phospho-T68, and HA panels are all from the same filter probed with anti-phospho-T68, stripped, and reprobed with anti-HA. Phospho-T26 (phospho-T68) and S28 are from an independent blot of portions of the same samples. In lanes 2 through 8, forms of HA-Chk2 were produced in the rabbit reticulocyte lysate system after incubation of the lysate with the designated concentration of vehicle control, caffeine, or wortmannin. Both caffeine and wortmannin inhibit Chk2 *trans*-phosphorylation and autophosphorylation. HA-Chk2 and its mutants were produced in either the in vitro translation wheat germ extract system or the in vitro translation reticulocyte lysate system, in which reticulocyte lysates were either mock treated or incubated with the indicated concentrations of caffeine or wortmannin. In lanes 9, 10, and 11, in vitro kinase assays were performed on the anti-HA immunoprecipitates in the presence of [γ-^{32}P]ATP only (lane 9) or in the presence of [γ-^{32}P]ATP and caffeine (10 mM, lane 10) or wortmannin (10 μM, lane 11) (top panel). IP, immunoprecipitation; IB, immunoblotting; ab, antibody. (C) Activation of Chk2 produced in wheat germ lysates. Phosphorylation of T68 and T26S28 was examined with Chk2 phospho-specific antibodies. HA-Chk2 was produced in the in vitro translation wheat germ extract system (lanes 1 to 3) or reticulocyte lysate system (lanes 4 and 5). HA-Chk2 was then precipitated with anti-HA antibody (lanes 2, 3, and 5) or mouse IgG (lanes 1 and 4). One Chk2 precipitate from the wheat germ extract system (lane 3) was incubated with reticulocyte lysate at 30°C for 30 min prior to the kinase assay. All the immunocomplexes were incubated with [γ-^{32}P]ATP for in vitro kinase assays. Recovery of Chk2 was monitored by immunoblotting with anti-HA (bottom panel) or with anti-phospho-T68 (middle panel) or by incorporation of [γ-^{32}P]ATP (top panel). IP, immunoprecipitation; IB, immunoblotting; ab, antibody.

quired for catalytic activation of Chk2 (32), and other autophosphorylation sites may also contribute to the mobility shift.

We analyzed a series of bacterially expressed GST-Chk2 alleles in order to identify domains in Chk2 that are required for kinase activity and domains that are subject to autophosphorylation (Fig. 3). In these assays, both autophosphorylation of Chk2 and *trans* phosphorylation of a GST-Cdc25C substrate peptide were monitored. Both of the kinase-defective alleles,

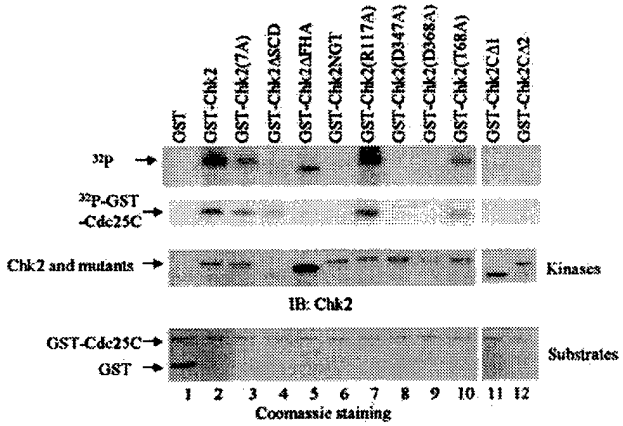


FIG. 3. Mutations in either SCD or FHA domain impair Chk2 kinase activities. GST fusion proteins were produced in bacteria, purified on glutathione beads, and released in soluble form with reduced glutathione. In vitro kinase assays were performed in the presence of Chk2 substrate GST-Cdc25C (amino acids 200 to 256) and [γ-^{32}P]ATP. Upper panel, [γ-^{32}P]ATP incorporation into GST-Chk2 and mutants; middle panel, [γ-^{32}P]ATP incorporation into GST-Cdc25C. Bottom panel, GST-Chk2 fusion proteins were quantified by immunoblotting with anti-Chk2 antibody (N-17). Coomassie brilliant blue staining showed substrate GST-Cdc25C. IB, immunoblotting.

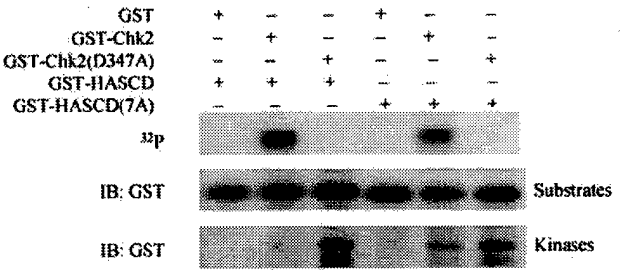


FIG. 4. Chk2 phosphorylates the SCD in vitro. In vitro kinase assays were performed on soluble GST-Chk2 and mutants in the presence of the substrate GST–HA-SCD or GST–HA-SCD(7A) and [γ-^{32}P]ATP. The top panel shows [γ-^{32}P]ATP incorporation into GST–HA-SCD or GST–HA-SCD(7A). GST–HA-SCD or GST–HA-SCD(7A) (middle panel). GST-Chk2 and its mutants (bottom panel) were detected by immunoblotting with anti-GST. IB, immunoblotting.

GST-Chk2(D347A) and GST-Chk2(D368A), lacked kinase activity in this assay. Within the SCD, the T68A substitution slightly reduced autophosphorylation and Cdc25C *trans*-phosphorylation activity. A more substantial decrease was observed with GST-Chk2(7A), in which all seven SQ/TQ sites within the SCD were replaced with AQ. Deletion of the entire SCD (GST-Chk2-ΔSCD) eliminated autophosphorylation, but only moderately diminished Cdc25 peptide phosphorylation. Similar results were observed with Chk2 and Chk2 mutants produced in the rabbit reticulocyte lysate system (data not shown). These data indicate that the SCD is required for maximal Chk2 kinase activity.

The stronger effects on autophosphorylation than on *trans* phosphorylation suggested that the SCD contains Chk2 autophosphorylation sites, including T68. Potential phosphorylation sites within the SCD include the seven SQ/TQ sites, as well as an additional 17 serines and 4 threonines. GST-Chk2 phosphorylated GST-SCD (Fig. 4, lane 3) and SCD(7A), lacking the SQ/TQ sites, to a lesser extent (Fig. 4, lane 5), but not GST (data not shown). A GST fusion protein of the Chk2 FHA domain was not phosphorylated (data not shown). Hence, the SCD is evidently a target for Chk2 autophosphorylation.

FHA domain is required for autophosphorylation of Chk2. FHA domains are phosphopeptide-binding modules (18, 19, 36, 37, 55, 57). Deletion of the core FHA domain of Chk2 resulted in a significantly lower autokinase activity and diminished *trans*-kinase activity (Fig. 3, lane 5). Similarly, triple mutation of the conserved FHA domain residues NGT (GST-Chk2-NGT) abrogated kinase activity (Fig. 3, lane 6). However, substitution of conserved R117 did not affect kinase activity. Similar results were obtained in the rabbit reticulocyte lysate system (data not shown). These data suggested that the FHA domain regulates Chk2 kinase activity, consistent with earlier work showing that mutations in the FHA domain prevent Chk2 activation in vivo (11, 32, 62).

Ionizing radiation enhances *ATM*-dependent oligomerization of Chk2. A yeast two-hybrid screen with yeast Rad53 as the bait identified Rad53 as an interacting protein (Z. Sun and D. F. Stern, unpublished results). Since this suggested that Rad53 forms dimers or oligomers, we determined whether Chk2 oligomers are produced in mammalian cells. In 293 cells expressing Flag-Chk2 and HA-Chk2, the tagged proteins coimmunoprecipitated, and the coimmunoprecipitation was enhanced by exposure to ionizing radiation (Fig. 5A and 5B). In *ATM*-deficient fibroblasts originating in a patient with ataxia-telangiectasia, enhancement of Chk2 oligomerization after gamma irradiation occurred with coexpression of wild-type Atm but not kinase-defective Atm (Fig. 5B). The higher baseline oligomerization compared to that in Fig. 5A probably reflects higher total expression of Chk2 with transient expression of both tagged proteins. These results demonstrated that at least two Chk2 molecules are components of an oligomer in vivo, that oligomerization is enhanced in response to DNA damage, and that this enhancement is *ATM* dependent. Thus, Chk2 oligomerization may be a regulated process that is linked to Chk2 activation.

Direct interactions of Chk2. Coimmunoprecipitation of two tagged Chk2 molecules may occur if they form homodimers or participate in a larger complex containing additional proteins. Bacterially expressed GST-Chk2 and kinase-defective GST-Chk2(D347A) interacted with His-Flag-Chk2 and His-Flag-Chk2(D347A) in GST pulldown experiments even though no other eukaryotic proteins were present (Fig. 6A and 6B). However, His-Flag-Chk2(D347A) does not interact well with GST-Chk2(D347A) (Fig. 6B). Hence, Chk2 homomers can form, provided that at least one molecule has catalytic activity.

We next determined if bacterially produced GST-Chk2 or kinase-defective GST-Chk2(D347A) would pull down HA-tagged Chk2 stably expressed in 293 cells. Both GST-Chk2 and GST-Chk2(D347A) preferentially bound to HA-Chk2 after gamma irradiation (Fig. 6C). Similar results were obtained with both GST pulldown and coimmunoprecipitation of wild-type and kinase-effective Chk2 in the rabbit reticulocyte lysate system (data not shown). In these experiments, with Chk2 produced in bacteria, rabbit reticulocyte lysates, or in mamma-

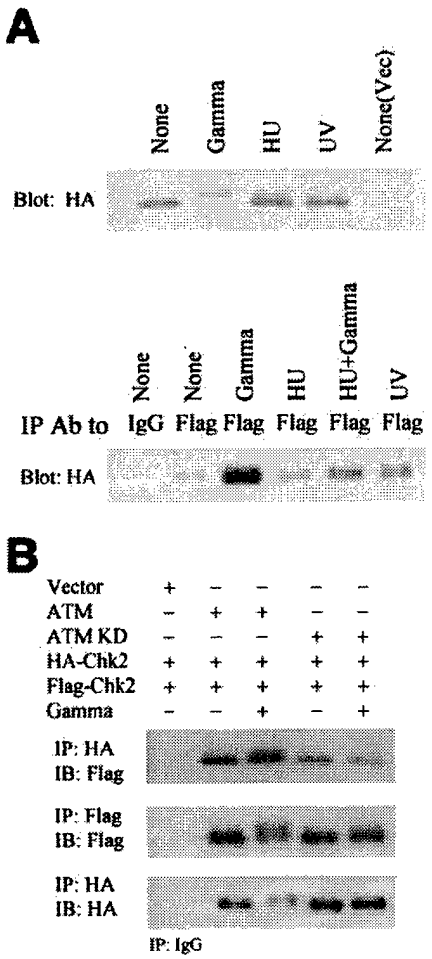


FIG. 5. Chk2 oligomerization. (A) 293 cells stably expressing Flag-Chk2 were transiently transfected with HA-Chk2. Transfectants were treated with 1 mM hydroxyurea for 24 h, beginning 24 h after transfection, or exposed to gamma irradiation (20 Gy) or UV (50 J/m^2) 48 h after transfection. Cell lysates were harvested 24 h after hydroxyurea treatment or 2 h after irradiation. Upper panel, expression and mobility shift of HA-Chk2 were determined by immunoblotting lysates with anti-HA antibody. Lower panel, coimmunoprecipitation with anti-Flag antibody was performed with equal portions of cell lysates and detected with anti-HA. (B) *ATM*-dependent increase in Chk2 coimmunoprecipitation. GM5849C ataxia-telangiectasia cells were transiently transfected with HA-Chk2 and Flag-Chk2, plus vector (pcDNA3), wild-type Flag-ATM, or kinase-defective Flag-ATMkd. Transfectants were exposed to 20 Gy of gamma irradiation 48 h after transfection. Cell lysates were harvested 2 h after irradiation. Equal amounts of lysates were immunoprecipitated with anti-HA (top and bottom panels) or anti-Flag (middle panel) antibodies. Precipitates were blotted for Flag-Chk2 (top two panels) or HA-Chk2 (bottom panel). IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

lian cells, kinase-defective Chk2 associated more strongly with Chk2 than did wild-type Chk2 (Fig. 6A and B and data not shown).

Chk2 oligomerization requires SCD and FHA domains. Domains required for Chk2 oligomerization were localized by mutational analysis. We first mapped the Chk2-interacting domain(s) by coimmunoprecipitation from 293 cells transiently expressing various forms of HA-tagged and Flag-tagged Chk2. We note that, at most, heterooligomers potentially represent only one-third of the total oligomerized Chk2 molecules (homooligomers of HA-tagged or Flag-tagged Chk2 or heterooligomers of HA-tagged and Flag-tagged Chk2). Deletion of the FHA domain virtually eliminated coimmunoprecipitation of Chk2 molecules.

Phosphorylation at T68 is induced by Atm and possibly Atr after DNA damage and is required for quantitative activation of Chk2 by ionizing radiation (1, 42, 64). Substitution of T68 with alanine only slightly reduced oligomerization both before and after gamma irradiation (Fig. 7A). Point substitution of other SQ/TQ sites (S19, T26/S28, S33/S35, and S50) did not significantly affect oligomerization (data not shown). However, mutation of all seven SQ/TQ sites, including T68, within the SCD significantly diminished coimmunoprecipitation (Fig. 7A). This suggests that Chk2 oligomerization involves one or more intact SQ/TQ sites within the SCD, but we cannot rule out a global structural effect of multiple substitutions. Deletion and substitution mutants of bacterially produced GST-Chk2 fusion proteins were used to determine the minimal fragment of Chk2 required for binding wild-type Chk2 produced in irradiated 293 cells (Fig. 7C). GST–HA-FHA was sufficient to preferentially bind wild-type Chk2 after DNA damage.

Since coimmunoprecipitation of two tagged Chk2 molecules required both the FHA domain and phosphorylation within the SCD, and since GST-FHA is sufficient to isolate Chk2 after DNA damage, we hypothesized that oligomerization of Chk2 is mediated by FHA/phospho-SCD interactions. Consistent with this hypothesis, GST–HA-FHA failed to pull down HA-Chk2ΔSCD in 293 cells after gamma irradiation. GST–HA-FHA did bind to HA-Chk2-SCD(7A) expressed in 293 cells after gamma irradiation (Fig. 7D), perhaps reflecting Chk2 autophosphorylation at non-SQ/TQ sites within the SCD (Fig. 4).

If an FHA domain in one Chk2 molecule associates with the phospho-SCD in another Chk2 molecule, then it should be possible to form a heterodimer containing one molecule with an intact phospho-SCD but deleted FHA domain, and another with a deleted SCD but intact FHA domain. Indeed, Flag-Chk2ΔSCD coimmunoprecipitated with HA-Chk2-ΔFHA, and the association was enhanced by ionizing radiation (Fig. 7B).

Chk2 oligomerization is phosphorylation dependent. In order to directly test the significance of phosphorylation on the putative FHA/phospho-SCD interaction, we determined whether the interaction is affected by phosphatase treatment. The ability of bacterially expressed GST-Chk2(D347A) to pull down bacterially produced His-Flag-Chk2 (Fig. 8A) was prevented by prior dephosphorylation of His-Flag-Chk2 with λ phosphatase (Fig. 8B). Similar results were obtained with the GST-FHA domain rather than full-length Chk2 as a pulldown reagent (Fig. 8B). In order to verify that this binding is mediated by the FHA domain rather than other sequences present in the fusion protein, we used GST fusion proteins containing and lacking the FHA domain and also GST1-221NGT, with alanine substitutions in a tripeptide important for FHA function. GST-Chk2 fusion proteins containing an intact FHA domain strongly bound to His-Flag-Chk2 but not His-Flag-Chk2(D347A). However, GST1-221NGT did not bind (Fig. 8C).

***trans* phosphorylation in the Chk2-Chk2 complex in vitro.** An important function of Chk2 oligomerization may be that it enables cross-phosphorylation of Chk2 molecules, which in

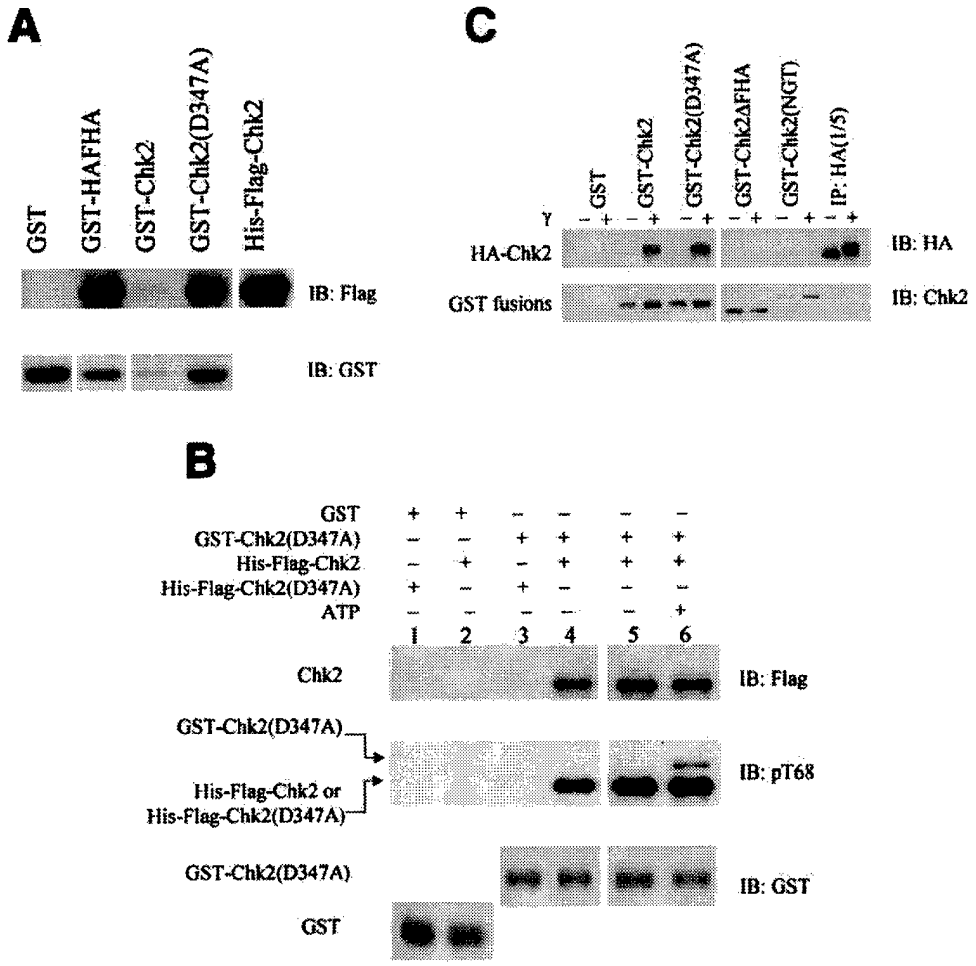


FIG. 6. Oligomerization of Chk2 produced in bacteria and 293 cells. (A) Bacterial expression. GST fusion protein pulldown experiment with bacterially produced soluble GST-HA-FHA, GST-Chk2, or kinase-defective GST-Chk2(D347A) incubated with His-Flag-Chk2. The pulldowns were blotted for wild-type His-Flag-Chk2 with anti-Flag antibody (top panel) and for total input of GST fusions with anti-GST antibodies (bottom panel). Images of different-sized GST fusion proteins in the bottom panel were cropped from one autoradiograph and aligned with one another to save space. (B) Phosphorylation of Chk2D347A by Chk2. Soluble GST-Chk2D347A produced in bacteria was used to pull down soluble His-Flag-Chk2 (lanes 4, 5, and 6) or kinase-defective His-Flag-Chk2(D347A) (lane 3). The affinity complexes were incubated in 1× kinase buffer in the absence (lane 5) or in the presence (lane 6) of ATP. Chk2 phosphorylation was evaluated by immunoblotting with anti-phospho-T68. GST-Chk2D347A is significantly larger than His-Flag-Chk2. (C) Mammalian expression. 293 cells stably expressing HA-Chk2 were exposed to 20 Gy of gamma irradiation. Cell lysates were harvested 2 h after irradiation. Lysates were normalized for protein concentration and used for pulldown with GST-Chk2 and its mutants. The GST pulldowns were blotted for HA-Chk2 with anti-HA antibody and for GST fusion protein with anti-Chk2 antibodies (N-17). Different sizes of GST fusions on the bottom panel were cropped and realigned from a single autoradiograph. IP, immunoprecipitation; IB, immunoblotting.

turn enhances Chk2 activation. Hence, we determined whether Chk2 could cross-phosphorylate a kinase-defective Chk2 molecule in a heterodimer. In vitro kinase assays with bacterially produced GST-Chk2(D347A) and His-Flag-Chk2 revealed that cross-phosphorylation of kinase-defective GST-Chk2(D347A) by His-Flag-Chk2 at least occurs at T68 (Fig. 6B).

Forced oligomerization of Chk2. We examined the functional consequences of Chk2 oligomerization in vivo by the regulated induction of dimerization. This system is based on the fact that the immunosuppressive drugs FK506 and rapamycin bind with high affinity to the cellular receptor FKBP12, which is an abundant, cytoplasmic 108-amino-acid protein (54). The synthetic ligand AP20187 binds with subnanomolar affinity to FKBPs with a single amino acid substitution, F36V (Fv), while binding with 1,000-fold lower affinity to the wild-type protein (14). Introduction of the FKBP (F36V) module into a heterologous protein allows ligand-dependent homo- and heterodimerization of the target proteins.

We introduced two tandem FKBP(F36V) modules into the amino terminus of wild-type or kinase-defective Chk2 (Fig. 1). When HEK 293 cells were cotransfected with both HA- and Flag-tagged 2FKBP-Chk2, approximately one-third of Flag-tagged 2FKBP-Chk2 coimmunoprecipitated with HA-tagged 2FKBP-Chk2 2 h after addition of the synthetic ligand (Fig. 9A). (Note that heterooligomerization between HA-tagged

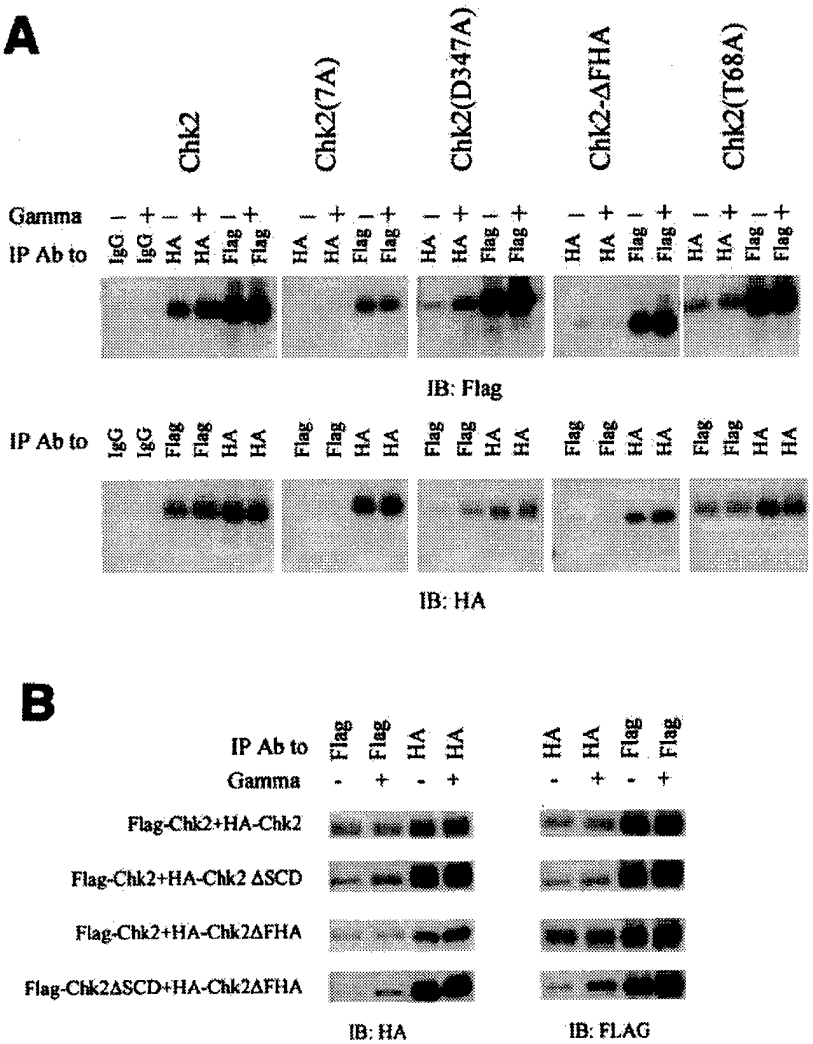
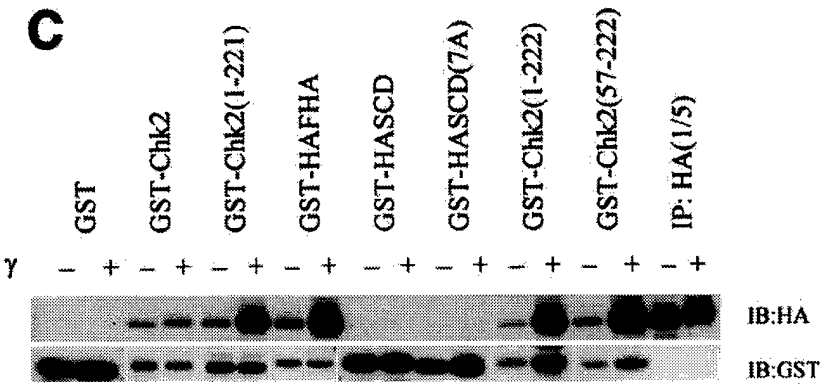
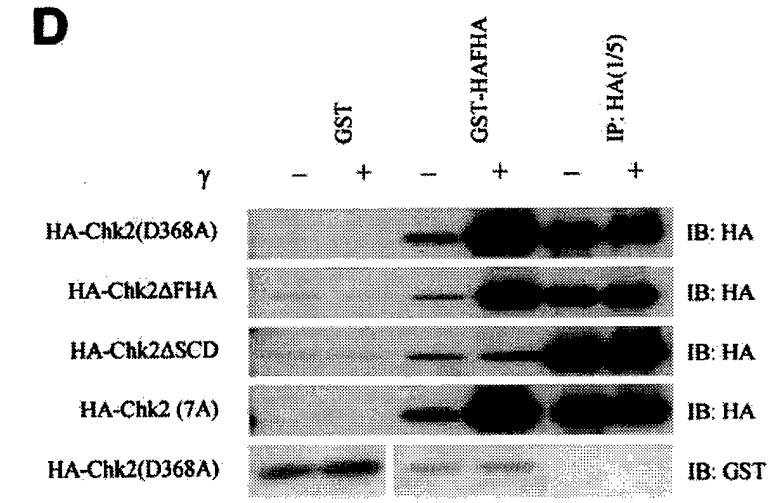


FIG. 7. Chk2 oligomerization domains. (A) Requirements for oligomerization in 293 cells. HA-tagged and Flag-tagged versions of individual Chk2 mutants were expressed by transient transfection in 293 cells. Transfectants were exposed to 20 Gy of gamma irradiation 48 h after transfection. Cell lysates were harvested 2 h after irradiation, and equal amounts were used for immunoprecipitation with anti-HA antibody or anti-Flag antibody as indicated and immunoblotted with anti-Flag (top panel) or anti-HA (bottom panel). Because homologous immunoprecipitations (e.g., IP anti-Flag, blot anti-Flag) were more efficient than heterologous coimmunoprecipitations (e.g., IP anti-Flag, blot anti-HA), only a one-fifth equivalent of homologous immunoprecipitation samples was analyzed relative to the cross-immunoprecipitation samples. (B) Oligomerization of FHA domain and SCD in vivo. Performed as in A except that Flag-tagged and HA-tagged Chk2 molecules were evaluated in the pairwise combinations listed at left. (C) GST-FHA binds to activated Chk2. Various Chk2-GST fusion proteins expressed in bacteria were used to isolate HA-Chk2 stably expressed in 293 cells. Experiments were performed as described in the legend to A. Equal portions of lysate from nonirradiated and irradiated cells were incubated with GST-Chk2 or its mutants. Pulldowns were blotted for HA-Chk2 with anti-HA antibody and for input of GST fusion protein with anti-GST antibodies. Different sizes of GST fusions on the bottom panel were cropped and realigned from one autoradiograph. Total lysate used for anti-HA immunoprecipitation was one-fifth of that for the GST pulldown. (D) Bacterially produced FHA domain of Chk2 binds to SCD in HA-Chk2 and its mutants expressed in 293 cells after gamma irradiation. The strategy described for Fig. 6C was used. Only one representative immunoblot for input of GST fusions (bottom panel) is shown. Different sizes of GST fusions on the bottom panel were cropped from one autoradiograph. (E) T68 phosphorylation of Chk2 and its mutants in vivo. Cells were handled essentially as described in A. Cell lysates were immunoprecipitated with anti-HA and detected by immunoblotting with anti-phospho-T68 or anti-HA. IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

and Flag-tagged 2FKBP-Chk2 potentially represents only one-third of the total amount of oligomerized 2FKBP-Chk2.) Ligand-induced oligomerization of Chk2 was significantly higher than that induced by ionizing irradiation (Fig. 9A). Induced oligomerization in vivo significantly increased T68 phosphorylation of wild-type Chk2 (Fig. 9B), reinforcing the conclusion that oligomerization facilitates Chk2 phosphorylation.

Surprisingly, kinase-defective Chk2 also showed greater T68 phosphorylation in the presence of ligand (Fig. 9B). Possible explanations include *trans* autophosphorylation in an oli-





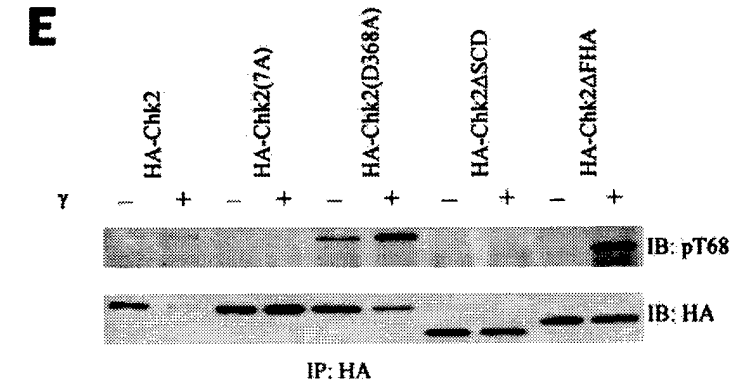
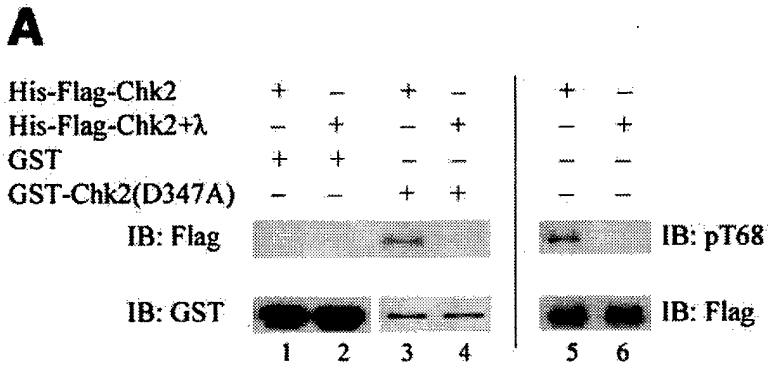
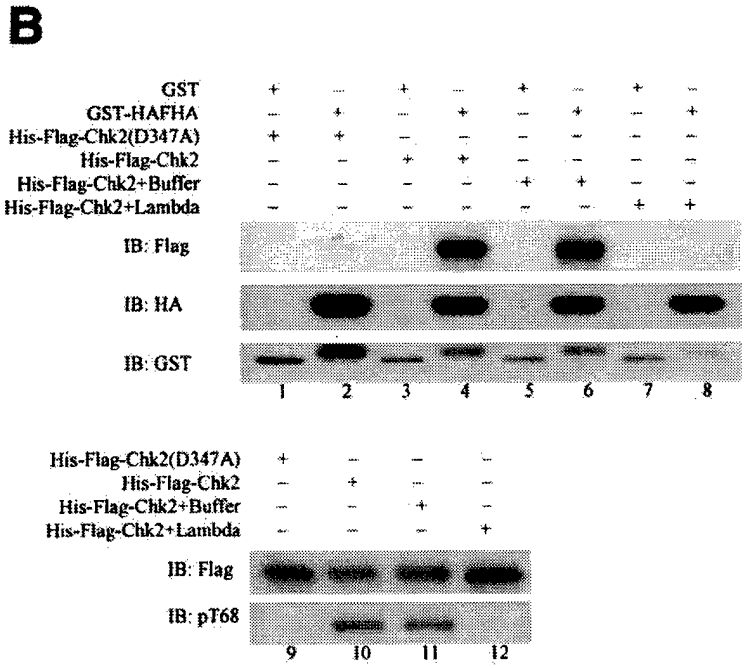
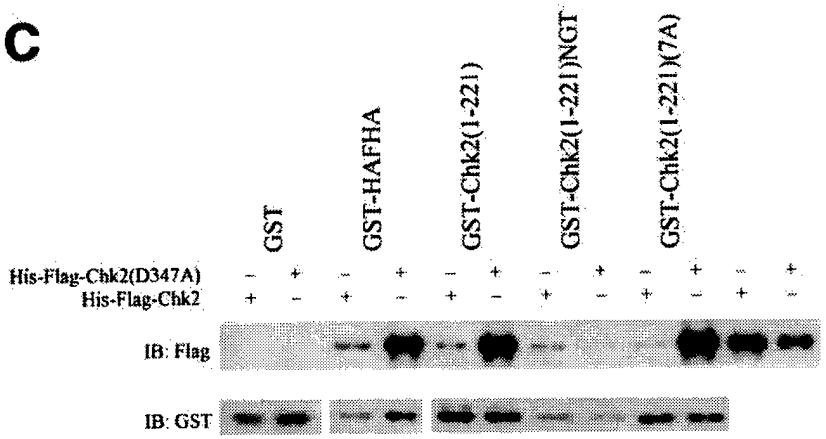


FIG. 7—*Continued*.

gomerized complex, in which kinase-defective Chk2 may associate with and be phosphorylated by the endogenous wild-type Chk2. Alternatively, the oligomerized Chk2 complex preferentially binds to and is phosphorylated by ATM/ATR kinases independent of Chk2 kinase activity. T68 phosphorylation in the ligand-induced oligomerized Chk2 immune complex was associated with increased Chk2 autokinase activity (Fig. 9C). However, this phosphorylation did not increase Chk2 *trans*


A
His-Flag-Chk2
His-Flag-Chk2+λ
GST
GST-Chk2(D347A)
IB: Flag
IB: GST
IB: pT68
IB: Flag
1 2 3 4 5 6


B
GST
GST-HAFHA
His-Flag-Chk2(D347A)
His-Flag-Chk2
His-Flag-Chk2+Buffer
His-Flag-Chk2+Lambda
IB: Flag
IB: HA
IB: GST
1 2 3 4 5 6 7 8
His-Flag-Chk2(D347A)
His-Flag-Chk2
His-Flag-Chk2+Buffer
His-Flag-Chk2+Lambda
IB: Flag
IB: pT68
9 10 11 12


C
GST
GST-HAFHA
GST-Chk2(1-221)
GST-Chk2(1-221)NGT
GST-Chk2(1-221)(7A)
His-Flag-Chk2(D347A)
His-Flag-Chk2
IB: Flag
IB: GST

phosphorylation of the Cdc25A polypeptide substrate (Fig. 9C), nor was it associated with measurable effects on cell cycle regulation or p53 stability (data not shown).

When Chk2 oligomerization was induced by ligand after exposure to a low dose of ionizing irradiation (2.5 Gy), both Chk2 auto- and *trans*-kinase activities increased (Fig. 9C) to levels comparable to that induced by 10-Gy irradiation (data not shown). This suggests that oligomerization plus a second DNA damage-dependent event, such as priming phosphorylation by ATM/ATR kinases, association with soluble activating or target proteins, or appropriate geometry of Chk2 oligomers induced by association with scaffolding molecules, is required for Chk2 activation and checkpoint pathway regulation.

DISCUSSION

We show here that Chk2 itself can phosphorylate Chk2 at T68 and other sites. Since T68 has already been identified as a *trans*-regulatory site, the Chk2-dependent phosphorylation of Chk2 has important implications for Chk2 regulation. Chk2 forms homomeric complexes in which the Chk2 FHA domain interacts with a second phosphorylated molecule of Chk2. Artificial dimerization of Chk2 in vivo concomitant with limited DNA damage augments Chk2 kinase activity. Finally, we provide evidence that Chk2 participates in DNA damage-dependent oligomeric complexes in vivo that have the same domain requirements as Chk2 homomers. These data suggest that the regulation of Chk2 by *trans* and autophosphorylation is more complicated than hitherto appreciated and involves a cascade of phosphorylation events that lead to the production of Chk2 homomeric complexes.

FHA domains are phosphopeptide interaction domains (18, 19, 36, 37, 55, 57). The Chk2 FHA domain is required for DNA damage-dependent Chk2 activation (11, 32, 62), and FHA domain mutations have been identified in alleles of Chk2 associated with variant $TP53^{+/+}$ Li-Fraumeni syndrome (5, 58) and a variety of tumors (27, 44). In the budding yeast DNA damage response, Rad53 FHA domains are required for DNA damage-dependent phosphorylation and activation of Rad53, which also requires the Atr homolog Mec1 and/or Tel1 (20, 47, 56, 57). A current model is that Mec1 is localized to sites of DNA damage and subsequently recruits and phosphorylates a second protein, Rad9, which is also required for damage-dependent activation of Rad53 (20, 47, 56, 57). Rad53 is recruited to the complex through interactions between Rad53 FHA domains and Rad9 phosphopeptides created by Mec1-mediated phosphorylation (M. F. Schwartz and D. F. Stern, submitted for publication). Since Mec1 is required for Rad53 activation and Rad53 has a cluster of potential PIKK phosphorylation sites similar to the Chk2 SCD, known to be a target for mammalian PIKKs, the relocalization of Rad53 mediated by FHA domains may be important for connecting Rad53 with the upstream activating PIKK Mec1. Another model, which is not incompatible with the first, is that dimerization of Rad53 through binding to dimeric Rad9 promotes Rad53 cross-phosphorylation and activation (22).

No mammalian Rad9 ortholog has been identified, nor has the binding partner for the Chk2 FHA domain. However, mutational analysis of Chk2 reveals the same dependencies of Chk2 function on the FHA domain (11, 32, 62), so that the same mechanisms probably operate in mammalian cells. With DNA damage, the initial function for the FHA domain will be coupling of Chk2 to upstream regulatory pathways, by bringing Chk2 to Atm/Atr and/or by adaptor- or scaffold-dependent activation. Our findings suggest that the FHA domain is required for an additional process involving intra- and/or intermolecular binding of the FHA domain to one or more phosphorylated sites within Chk2.

Similar to the Rad53 FHA domains, the Chk2 FHA domain is required for its PIKK-dependent phosphorylation. Point mutation (R145W) (33, 62) or deletion mutation (21 and 42 amino acids surrounding R145) (33) of the FHA domain abolished T68 phosphorylation. This suggests that the FHA domain couples Chk2 to an Atm- or Atr-activated complex. Nevertheless, we observed that deletion of the core FHA domain (amino acids 115 to 175) of Chk2 spared T68 phosphorylation after gamma irradiation (Fig. 7E) and binding to the bacterially produced FHA domain (Fig. 7D, second panel). This suggests that an additional mechanism of T68 phosphorylation bypassing the requirement for the Chk2 FHA domain can operate under some circumstances.

T68 is phosphorylated during damage-dependent activation of Chk2 in vivo and is likely to be a target for both Atm and Atr (1, 41, 42, 64). Phosphorylation at T68 is permissive for further Chk2 autophosphorylation at two sites in the activation loop, which is thought to be required for full activation of Chk2 (33). The mechanism by which T68 phosphorylation promotes Chk2 activation has not been determined. It may promote allosteric changes that are permissive for activation loop phosphorylation or may regulate intra- or intermolecular Chk2 interactions. Our finding that deletion of the FHA domain impairs the kinase activity of bacterially produced Chk2 suggests that this

FIG. 8. Phosphorylation-dependent oligomerization of Chk2. (A) Bacterially expressed His-Flag-Chk2 was incubated in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of λ phosphatase and either blotted directly with anti-phospho-T68 or anti-Flag (lanes 5 and 6) or pulled down with either GST or GST-Chk2(D347A) expressed in bacteria (lanes 1, 2, 3, and 4). Affinity-purified Chk2 was detected by blotting with anti-Flag, and the loading of GST proteins was monitored with anti-GST. (B) Phosphorylation-dependent binding of the Chk2 FHA domain. Bacterially expressed His-Flag-Chk2 or His-Flag-Chk2(D347A) was incubated in the absence (lanes 5, 6, and 11) or presence (lanes 7, 8, and 12) of λ phosphatase and immunoblotted with anti-Flag or anti-phospho-T68 (lanes 9, 10, 11, and 12). Bacterially produced GST or GST–HA-FHA was used to pull down additional portions of phosphatase-treated or nontreated kinase-defective His-Flag-Chk2(D347A) or His-Flag-Chk2. Wild-type and kinase-defective His-Flag-Chk2 were detected with anti-Flag antibody (upper panel), for input of GST–HA-FHA with anti-HA antibody (second panel) and for GST with anti-GST antibody (lower panel). (C) Effect of FHA domain mutations on binding to His-Flag-Chk2. GST fusion proteins expressed in bacteria were used to pull down bacterially produced wild-type and kinase-defective His-Flag-Chk2. His-Flag-tagged Chk2 was detected with anti-Flag antibody, and GST fusions were detected with anti-GST. Differently sized GST fusion proteins on the bottom panel were cropped and realigned from one autoradiograph. IP, immunoprecipitation; IB, immunoblotting.

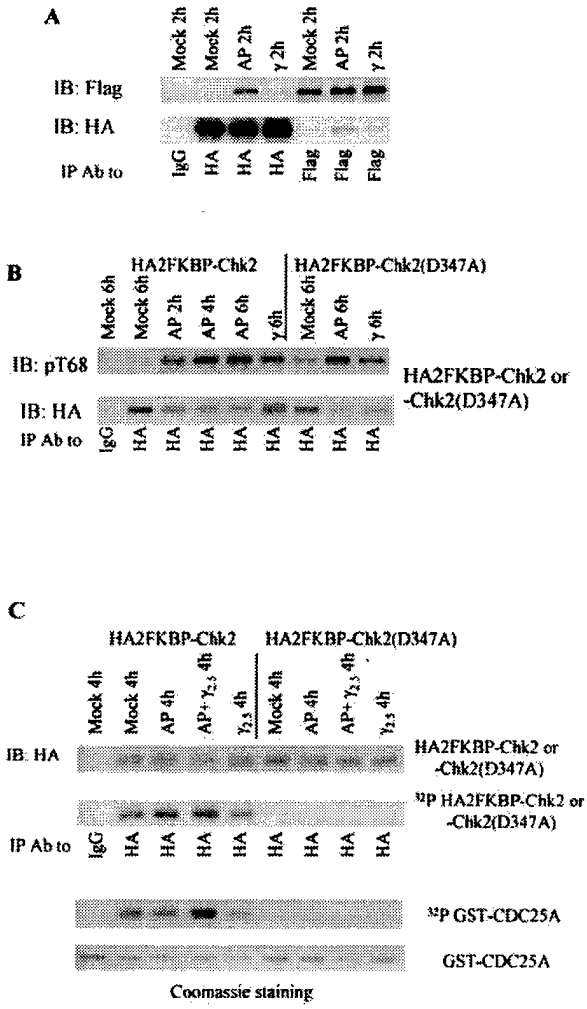


FIG. 9. Forced oligomerization and Chk2 activation. (A) AP20187 induced Chk2 oligomerization in vivo. HEK 293 cells were transiently cotransfected with both HA- and Flag-tagged 2FKBP-Chk2. Transfectants were either mock treated, treated with 10 μM AP20187, or irradiated with 10 Gy of gamma irradiation 48 h after transfection. Lysates were harvested 2 h after treatment and used for immunoprecipitation followed by immunoblotting. (B) Induced oligomerization resulted in T68 phosphorylation. Performed as in A, except that only HA-tagged wild-type or kinase-defective 2FKBP-Chk2 was transiently expressed. Lysates were harvested at each time point as indicated after treatment and used for immunoprecipitation with anti-HA antibody followed by immunoblotting for anti-phospho-T68 (top panel) or anti-HA (bottom panel, duplicate blot) antibodies. (C) Induced oligomerization after exposure to low-dose ionizing irradiation activated Chk2. Performed as in B, except that the dose of γ irradiation was 2.5 Gy and AP20187 was added to one set of 293 cells immediately after exposure to 2.5 Gy of irradiation. Immunocomplexes were incubated with [γ-^{32}P]ATP in the presence of bacterially produced GST-Cdc25A (amino acids 101 to 140). Recovery of Chk2 was monitored by immunoblotting with anti-HA (top panel) or incorporation of [γ-^{32}P]ATP (second panel). Recovery of GST-Cdc25A was detected by incorporation of [γ-^{32}P]ATP (third panel) or Coomassie staining (bottom panel). IP, immunoprecipitation; IB, immunoblotting; Ab, antibody.

domain has a positive regulatory influence. However, we cannot rule out the possibility that this is a result of nonspecific effects on Chk2 folding.

An important implication of the finding that Chk2 can phosphorylate itself at a known regulatory site is that phospho-Chk2 may be able to activate other molecules of Chk2. In this scenario, DNA damage-dependent activation of PIKKs would result in phosphorylation of Chk2 at T68 and other SQ/TQ sites within the SCD (1, 41, 42, 64). Phosphorylation of T68 would enable Chk2 autophosphorylation at the activation loop (32) and additional sites within the SCD. Nonphosphorylated Chk2 molecules would then be recruited and activated through FHA-SCD interactions and cross-phosphorylation. Hence, after priming phosphorylation by PIKKs, additional molecules could be activated independent of PIKK activity, thereby latching on Chk2 activation. This model is consistent with the intriguing finding that Rad9 can activate Rad53 in an ATP-dependent manner in the absence of Mec1 (22). Scaffolding of a Rad53 dimer by Rad9 could promote priming activation of Rad53, which could then activate additional molecules of Rad53. The catalytic function of Rad53 would be the source of the ATP dependence of the activation.

The ability of the Chk2 FHA domain to bind phospho-SCD suggests that FHA/phospho-SCD interactions may be important in intra- or intermolecular regulation of Chk2. Phosphorylation of the Chk2 SCD may enable an intramolecular interaction with the FHA domain that would either disturb a basal occupation of the kinase domain by the FHA domain or induce a structural change in the kinase domain, which is directly adjacent to the FHA domain. Alternatively, FHA/phospho-SCD interactions may enable recruitment of a second molecule of Chk2 through its FHA domain to a complex, thereby facilitating catalytic activation through cross-phosphorylation.

In other signaling systems involving phosphopeptide binding interactions, dynamic changes in association and localization of signaling proteins are mediated by a cascade of phosphopeptide/binding domain interactions. STATs are recruited to activated receptor tyrosine kinases by interaction of their SH2 domains with phosphopeptides on the phosphorylated receptors. Once recruited, the receptors phosphorylate the STATs, creating new STAT SH2 binding sites on the STATs themselves. An exchange of STAT-SH2/phosphoreceptor interactions with STAT-SH2/phospho-STAT interactions is an important step in releasing STATs from the sites of activation at the cell surface for transit to the nucleus, where they function as transcription factors (4, 13, 17).

Similarly, the R-Smad MH2 domain, a phosphoserine-binding motif structurally related to FHA domains, binds to phosphopeptides on the TGF-β-activated TGF-β type I receptor. Phosphorylation at the C-terminal serine residues of R-Smad by TGF-β type I receptor promotes homooligomerization by binding to the MH2 domain of the second R-Smad molecule and then dissociation from TGF-β type I receptor (46, 51, 61). It is noteworthy that unphosphorylatable co-Smad Smad4 competes with a phospho-R-Smad homooligomeric complex to form a more stable Smad4/phospho-R-Smad heterooligomeric complex (46, 61).

The ability of the Chk2 FHA domain to bind phospho-Chk2 as well as the likelihood that it binds to putative upstream activating proteins suggest a similar series of phosphopeptide binding site

exchanges in Chk2 activation. An overall model is that DNA checkpoint activation results in direct recruitment of PIKKs (ATR and ATM) and, independently, the Rad1-Rad9 (unrelated to budding yeast Rad9)-Hus1PCNA-related complex to sites of DNA damage (15, 31, 43, 66). DNA damage promotes relocalization of Chk2 phosphorylated at T68 (60). Recruitment of Chk2 would depend upon an interaction between its FHA domain and a phosphorylated constituent of the DNA damage complex. Once recruited, Chk2 would be activated by PIKK-dependent phosphorylation and/or by scaffolding-induced oligomerization. Additional Chk2 molecules would be activated similarly and by FHA domain-dependent binding of additional Chk2 molecules to already active and phosphorylated Chk2. Head-to-tail polymerization of phospho-Chk2 could potentially result in assembly of large active complexes.

Dissociation of Chk2 from the activated complexes could result from competition among FHA domain binding sites. We have found that phospho-Rad9 complexes with kinase-defective Rad53 are more abundant than those between phospho-Rad9 and kinase-active Rad53 (M. F. Schwartz and D. F. Stern, unpublished data). These data may be explained by a phosphorylation-dependent mechanism for dissociation of Chk2 FHA domain complexes that have already formed. If, hypothetically, the Chk2 FHA domain interacts more strongly with the Chk2 phospho-SCD than with the Rad9-like binding target in DNA damage complexes, then progressive activation of Chk2 would favor production of Chk2 homodimers over heteromers with the activation complex. This would result in release of active Chk2 homodimers, in much the same way that phosphorylated STATs and R-Smads are released from the activating receptors. Since heteromeric complexes between kinase-active Chk2 and inactive Chk2 are more readily isolated than those between two kinase-active molecules, even with bacterially expressed proteins, it is possible that the dual phosphorylated dimer is itself unstable and dissociates to release active monomers.

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ADDENDUM IN PROOF

Related work is reported by Ahn et al. (J. Y. Ahn, X. Li, H. L. Davis, and C. E. Canman, J. Biol. Chem., in press).

REFERENCES

1. **Ahn, J. Y., J. K. Schwarz, H. Piwnica-Worms, and C. E. Canman.** 2000. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. Cancer Res. **60**:5934–5936.
2. **Anderson, C. W., and S. P. Lees-Miller.** 1992. The nuclear serine/threonine protein kinase DNA-PK. Crit. Rev. Eukaryot. Gene Expr. **2**:283–314.
3. **Banin, S., L. Moyal, S. Shieh, Y. Taya, C. W. Anderson, L. Chessa, N. I. Smorodinsky, C. Prives, Y. Reiss, Y. Shiloh, and Y. Ziv.** 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science **281**:1674–1677.
4. **Becker, S., B. Groner, and C. W. Muller.** 1998. Three-dimensional structure of the Stat3beta homodimer bound to DNA. Nature **394**:145–151.
5. **Bell, D. W., J. M. Varley, T. E. Szydlo, D. H. Kang, D. C. Wahrer, K. E. Shannon, M. Lubratovich, S. J. Verselis, K. J. Isselbacher, J. F. Fraumeni, J. M. Birch, F. P. Li, J. E. Garber, and D. A. Haber.** 1999. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science **286**:2528–2531.
6. **Blasina, A., I. V. de Weyer, M. C. Laus, W. H. Luyten, A. E. Parker, and C. H. McGowan.** 1999. A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr. Biol. **9**:1–10.
7. **Brown, A. L., C. H. Lee, J. K. Schwarz, N. Mitiku, H. Piwnica-Worms, and J. H. Chung.** 1999. A human Cds1-related kinase that functions downstream of ATM protein in the cellular response to DNA damage. Proc. Natl. Acad. Sci. USA **96**:3745–3750.
8. **Canman, C. E., D. S. Lim, K. A. Cimprich, Y. Taya, K. Tamai, K. Sakaguchi, E. Appella, M. B. Kastan, and J. D. Siliciano.** 1998. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science **281**:1677–1679.
9. **Chan, D. W., S. C. Son, W. Block, R. Ye, K. K. Khanna, M. S. Wold, P. Douglas, A. A. Goodarzi, J. Pelley, Y. Taya, M. F. Lavin, and S. P. Lees-Miller.** 2000. Purification and characterization of ATM from human placenta. A manganese-dependent, wortmannin-sensitive serine/threonine protein kinase. J. Biol. Chem. **275**:7803–7810.
10. **Chaturvedi, P., W. K. Eng, Y. Zhu, M. R. Mattern, R. Mishra, M. R. Hurle, X. Zhang, R. S. Annan, Q. Lu, L. F. Faucette, G. F. Scott, X. Li, S. A. Carr, R. K. Johnson, J. D. Winkler, and B. B. Zhou.** 1999. Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Oncogene **18**:4047–4054.
11. **Chehab, N. H., A. Malikzay, M. Appel, and T. D. Halazonetis.** 2000. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev. **14**:278–288.
12. **Chehab, N. H., A. Malikzay, E. S. Stavridi, and T. D. Halazonetis.** 1999. Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc. Natl. Acad. Sci. USA **96**:13777–13782.
13. **Chen, X., U. Vinkemeier, Y. Zhao, D. Jeruzalmi, J. E. Darnell, Jr., and J. Kuriyan.** 1998. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. Cell **93**:827–839.
14. **Clackson, T., W. Yang, L. W. Rozamus, M. Hatada, J. F. Amara, C. T. Rollins, L. F. Stevenson, S. R. Magari, S. A. Wood, N. L. Courage, X. Lu, F. Cerasoli, Jr., M. Gilman, and D. A. Holt.** 1998. Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. Proc. Natl. Acad. Sci. USA **95**:10437–10442.
15. **Cortez, D., S. Guntuku, J. Qin, and S. J. Elledge.** 2001. ATR and ATRIP: partners in checkpoint signaling. Science **294**:1713–1716.
16. **Cortez, D., Y. Wang, J. Qin, and S. J. Elledge.** 1999. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science **286**:1162–1166.
17. **Darnell, J. E., Jr.** 1997. STATs and gene regulation. Science **277**:1630–1635.
18. **Durocher, D., J. Henckel, A. R. Fersht, and S. P. Jackson.** 1999. The FHA domain is a modular phosphopeptide recognition motif. Mol. Cell **4**:387–394.
19. **Durocher, D., I. A. Taylor, D. Sarbassova, L. F. Haire, S. L. Westcott, S. P. Jackson, S. J. Smerdon, and M. B. Yaffe.** 2000. The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. Mol. Cell **6**:1169–1182.
20. **Emili, A.** 1998. MEC1-dependent phosphorylation of Rad9p in response to DNA damage. Mol. Cell **2**:183–189.
21. **Falck, J., N. Mailand, R. G. Syljuasen, J. Bartek, and J. Lukas.** 2001. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature **410**:842–847.
22. **Gilbert, C. S., C. M. Green, and N. F. Lowndes.** 2001. Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. Mol. Cell **8**:129–136.
23. **Hartwell, L. H., and M. B. Kastan.** 1994. Cell cycle control and cancer. Science **266**:1821–1828.
24. **Haruki, N., H. Saito, Y. Tatematsu, H. Konishi, T. Harano, A. Masuda, H. Osada, Y. Fujii, and T. Takahashi.** 2000. Histological type-selective, tumor-predominant expression of a novel CHK1 isoform and infrequent in vivo somatic CHK2 mutation in small cell lung cancer. Cancer Res. **60**:4689–4692.
25. **Hirao, A., Y. Y. Kong, S. Matsuoka, A. Wakeham, J. Ruland, H. Yoshida, D. Liu, S. J. Elledge, and T. W. Mak.** 2000. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science **287**:1824–1827.
26. **Hofmann, K., and P. Bucher.** 1995. The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. Trends Biochem. Sci. **20**:347–349.
27. **Hofmann, W. K., C. W. Miller, K. Tsukasaki, S. Tavor, T. Ikezoe, D. Hoelzer, S. Takeuchi, and H. P. Koeffler.** 2001. Mutation analysis of the DNA-damage checkpoint gene CHK2 in myelodysplastic syndromes and acute myeloid leukemias. Leukoc. Res. **25**:333–338.
28. **Inbal, B., G. Shani, O. Cohen, J. L. Kissil, and A. Kimchi.** 2000. Death-

associated protein kinase-related protein 1, a novel serine/threonine kinase involved in apoptosis. Mol. Cell. Biol. **20**:1044–1054.
29. **Khanna, K. K.** 2000. Cancer risk and the ATM gene: a continuing debate. J. Natl. Cancer Inst. **92**:795–802.
30. **Kim, S. T., D. S. Lim, C. E. Canman, and M. B. Kastan.** 1999. Substrate specificities and identification of putative substrates of ATM kinase family members. J. Biol. Chem. **274**:37538–37543.
31. **Kondo, T., T. Wakayama, T. Naiki, K. Matsumoto, and K. Sugimoto.** 2001. Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms. Science **294**:867–870.
32. **Lee, C. H., and J. H. Chung.** 2001. The hCds1 (Chk2)-FHA domain is essential for a chain of phosphorylation events on hCds1 that is induced by ionizing radiation. J. Biol. Chem. **276**:30537–30541.
33. **Lee, J. S., K. M. Collins, A. L. Brown, C. H. Lee, and J. H. Chung.** 2000. hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response. Nature **404**:201–204.
34. **Li, J., G. I. Lee, S. R. Van Doren, and J. C. Walker.** 2000. The FHA domain mediates phosphoprotein interactions. J. Cell Sci. **113**:4143–4149.
35. **Li, J., G. P. Smith, and J. C. Walker.** 1999. Kinase interaction domain of kinase-associated protein phosphatase, a phosphoprotein-binding domain. Proc. Natl. Acad. Sci. USA **96**:7821–7826.
36. **Liao, H., L. J. Byeon, and M. D. Tsai.** 1999. Structure and function of a new phosphopeptide-binding domain containing the FHA2 of Rad53. J. Mol. Biol. **294**:1041–1049.
37. **Liao, H., C. Yuan, M. I. Su, S. Yongkiettrakul, D. Qin, H. Li, I. J. Byeon, D. Pei, and M. D. Tsai.** 2000. Structure of the FHA1 domain of yeast Rad53 and identification of binding sites for both FHA1 and its target protein Rad9. J. Mol. Biol. **304**:941–951.
38. **Madhani, H. D.** 2001. Accounting for specificity in receptor tyrosine kinase signaling. Cell **106**:9–11.
39. **Massague, J., and Y. G. Chen.** 2000. Controlling TGF-beta signaling. Genes Dev. **14**:627–644.
40. **Matsuoka, S., M. Huang, and S. J. Elledge.** 1998. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science **282**:1893–1897.
41. **Matsuoka, S., G. Rotman, A. Ogawa, Y. Shiloh, K. Tamai, and S. J. Elledge.** 2000. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc. Natl. Acad. Sci. USA **97**:10389–10394.
42. **Melchionna, R., X. B. Chen, A. Blasina, and C. H. McGowan.** 2000. Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. Nat. Cell Biol. **2**:762–765.
43. **Melo, J. A., J. Cohen, and D. P. Toczyski.** 2001. Two checkpoint complexes are independently recruited to sites of DNA damage in vivo. Genes Dev. **15**:2809–2821.
44. **Miller, C. W., T. Ikezoe, U. Krug, W. K. Hofmann, S. Tavor, V. Vegesna, K. Tsukasaki, S. Takeuchi, and H. P. Koeffler.** 2002. Mutations of the CHK2 gene are found in some osteosarcomas, but are rare in breast, lung, and ovarian tumors. Genes Chromosomes Cancer **33**:17–21.
45. **Pellicioli, A., C. Lucca, G. Liberi, F. Marini, M. Lopes, P. Plevani, A. Romano, P. P. Di Fiore, and M. Foiani.** 1999. Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. EMBO J. **18**:6561–6572.
46. **Qin, B. Y., B. M. Chacko, S. S. Lam, M. P. de Caestecker, J. J. Correia, and K. Lin.** 2001. Structural basis of Smad1 activation by receptor kinase phosphorylation. Mol. Cell **8**:1303–1312.
47. **Sanchez, Y., B. A. Desany, W. J. Jones, Q. Liu, B. Wang, and S. J. Elledge.** 1996. Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science **271**:357–360.
48. **Sarkaria, J. N., E. C. Busby, R. S. Tibbetts, P. Roos, Y. Taya, L. M. Karnitz, and R. T. Abraham.** 1999. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res. **59**:4375–4382.
49. **Sarkaria, J. N., R. S. Tibbetts, E. C. Busby, A. P. Kennedy, D. E. Hill, and R. T. Abraham.** 1998. Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res. **58**:4375–4382.
49a. **Schwartz, M. F., J. K. Duong, Z. Sun, J. S. Morrow, D. Pradhan, and D. F. Stern.** Rad9 phosphorylation sites couple Rad53 to the *Saccharomyces cerevisiae* DNA damage checkpoint. Mol. Cell. in press.
50. **Shani, G., S. Henis-Korenblit, G. Jona, O. Gileadi, M. Eisenstein, T. Ziv, A. Admon, and A. Kimchi.** 2001. Autophosphorylation restrains the apoptotic activity of DRP-1 kinase by controlling dimerization and calmodulin binding. EMBO J. **20**:1099–1113.
51. **Shi, Y.** 2001. Structural insights on Smad function in TGFbeta signaling. Bioessays **23**:223–232.
52. **Shieh, S. Y., J. Ahn, K. Tamai, Y. Taya, and C. Prives.** 2000. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev. **14**:289–300.
53. **Sodha, N., R. Williams, J. Mangion, S. L. Bullock, M. R. Yuille, and R. A. Eeles.** 2000. Screening hCHK2 for mutations. Science **289**:359.
54. **Spencer, D. M., T. J. Wandless, S. L. Schreiber, and G. R. Crabtree.** 1993. Controlling signal transduction with synthetic ligands. Science **262**:1019–1024.
55. **Stone, J. M., M. A. Collinge, R. D. Smith, M. A. Horn, and J. C. Walker.** 1994. Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase. Science **266**:793–795.
56. **Sun, Z., D. S. Fay, F. Marini, M. Foiani, and D. F. Stern.** 1996. Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. **10**:395–406.
57. **Sun, Z., J. Hsiao, D. S. Fay, and D. F. Stern.** 1998. Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Science **281**:272–274.
58. **Vahteristo, P., A. Tamminen, P. Karvinen, H. Eerola, C. Eklund, L. A. Aaltonen, C. Blomqvist, K. Aittomaki, and H. Nevanlinna.** 2001. p53, CHK2, and CHK1 genes in Finnish families with Li-Fraumeni syndrome: further evidence of CHK2 in inherited cancer predisposition. Cancer Res. **61**:5718–5722.
59. **Vialard, J. E., C. S. Gilbert, C. M. Green, and N. F. Lowndes.** 1998. The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. EMBO J. **17**:5679–5688.
60. **Ward, I. M., X. Wu, and J. Chen.** 2001. Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. J. Biol. Chem. **276**:47755–47758.
61. **Wu, J. W., M. Hu, J. Chai, J. Seoane, M. Huse, C. Li, D. J. Rigotti, S. Kyin, T. W. Muir, R. Fairman, J. Massague, and Y. Shi.** 2001. Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signaling. Mol. Cell **8**:1277–1289.
62. **Wu, X., S. R. Webster, and J. Chen.** 2001. Characterization of tumor-associated Chk2 mutations. J. Biol. Chem. **276**:2971–2974.
63. **Xu, X., J. Liao, K. E. Creek, and L. Pirisi.** 1999. Human keratinocytes and tumor-derived cell lines express alternatively spliced forms of transforming growth factor-alpha mRNA, encoding precursors lacking carboxyl-terminal valine residues. Oncogene **18**:5554–5562.
64. **Zhou, B. B., P. Chaturvedi, K. Spring, S. P. Scott, R. A. Johanson, R. Mishra, M. R. Mattern, J. D. Winkler, and K. K. Khanna.** 2000. Caffeine abolishes the mammalian G_2/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. J. Biol. Chem. **275**:10342–10348.
65. **Zhou, B. B., and S. J. Elledge.** 2000. The DNA damage response: putting checkpoints in perspective. Nature **408**:433–439.
66. **Zou, L., D. Cortez, and S. J. Elledge.** 2002. Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev. **16**:198–208.